

Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 23-35 and 37-78 are pending in the application, with 23, 40, 51, 66, 75 and 77 being the independent claims. Claim 36 is sought to be cancelled without prejudice to or disclaimer of the subject matter therein. Claims 29, 30, 32, 33, 55, 56, 58 and 59 are sought to be amended. Support for the amendments can be found in the claims as originally filed and throughout the specification. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

I. Election / Restriction

The Examiner acknowledged Applicants' election with traverse of Group I, claims 23-74. It is the Examiner's position that the requirement is still deemed proper and therefore made it final. (*See Office Action, page 2.*)

Applicants note that the claims of Group I, drawn to an antibody that binds to a protein whose sequence is shown in SEQ ID NO:2 and/or which is encoded by the cDNA contained in ATCC Deposit Number 209003, and the claims of Group II, drawn to a method of screening for a compound which binds to a protein whose sequence is

shown in SEQ ID NO:2 and/or which is encoded by the cDNA contained in ATCC Deposit Number 209003, are related as between a product (Group I) and a process for using the product (Group II). Further, the process claims of Group II depend from and include all the limitations of the product claims of Group I.

In light of the decisions in *In re Ochiai*, 71 F.3d 1565, 37 USPQ2d 1127 (Fed. Cir. 1995) and *In re Brouwer*, 77 F.3d 422, 37 USPQ2d 1663 (Fed. Cir. 1996), a notice was published in the Official Gazette which set forth new guidelines for the treatment of product and process claims. See 1184 OG 86 (March 26, 1996). Specifically, the notice states that

in the case of an elected product claim, *rejoinder will be permitted when a product claim is found allowable* and the withdrawn process claim depends from or otherwise includes all the limitations of an allowed product claim.

Id. (emphasis added). Accordingly, if the elected claims of Group I *are found allowable*, Applicants respectfully request that the claims of Group II be rejoined and examined for patentability based on the reasons discussed above.

II. Objections to the Specification

The Examiner withdrew the objections to the specification. (See Office Action, page 3.) Applicants thank the Examiner for the withdrawal of these objections.

III. Rejections under 35 U.S.C. § 112, Second Paragraph

The Examiner withdrew the rejection of claims 39, 50, 65 and 74 under 35 U.S.C. § 112, second paragraph, in view of Applicants' arguments. (See Office Action, page 3.) Applicants thank the Examiner for the withdrawal of these rejections.

IV. Rejections under 35 U.S.C. § 101

The Examiner maintained the rejection of claims 23-74 under 35 U.S.C. § 101 as allegedly "not supported by either a specific and substantial asserted utility or a well established utility." (Office Action, page 3.) According to the Examiner,

as was set forth in *Brenner v. Manson*, 383 U.S. 519 (1966), the instant invention lacks a specific and substantial real world utility absent elucidation of the biological function of the disclosed protein agonist which the claimed antibody is directed and any role that the antibodies identified as modulators of the protein would play in modulation or identification of any disease state associated with that biological function. Without further research and experimentation, the claimed antibodies do not provide an immediate benefit to the public.

(Office Action, pages 4-5.)

A. The Examiner's Reliance on *Brenner v. Manson* is Misguided

Applicants respectfully emphasize that the specification *does* disclose at least one specific and substantial utility of the EBI-2 G-protein coupled receptor. Moreover, it is apparent that the instant case is *not* analogous to the situation in *Brenner v. Manson*, contrary to the Examiner's implication.

In *Brenner*, the issue was not whether a disclosed utility was sufficient. Rather, the applicant was trying to establish an earlier date of invention for the purpose of

provoking an interference. 383 U.S. at 521. Indeed, the examiner's initial basis for refusing to declare an interference was that the applicant had *failed to disclose any utility* at all. *Id.* at 521. Thus, the issue in *Brenner* was whether the applicant had made an adequate "showing" to establish a prior date of invention, *i.e.*, whether "the process claim has been reduced to production of a product shown to be useful" through actual demonstration of the utility. *Id.* at 534. The only evidence offered by the applicant to make this showing was a reference to an article by a third party showing the activity of an adjacent homologue of the subject steroid compound. *See id.* at 521-522. The appellate court agreed that the applicant had done nothing to show or demonstrate that the compound was indeed useful. *See id.* at 521. Thus, it upheld the rejection of the request for declaration of an interference. *Id.* at 536.

In contrast, the issue in the present case is whether the instant application explicitly teaches at least one utility that meets the requirements of § 101. Applicants submit that the specification discloses a number of specific uses for EBI-2 G-protein coupled receptor molecules. Further, the Federal Circuit has recently articulated the standard for utility in light of *Brenner*:

The threshold of utility is not high: An invention is "useful" under section 101 if it is capable of providing some identifiable benefit. *See Brenner v. Manson*, 383 U.S. 519, 534 (1996); *Brooktree Corp. v. Advanced Micro Devices, Inc.*, 977 F.2d 1555, 1571 (Fed. Cir. 1992) ("To violate § 101 the claimed device must be totally incapable of achieving a useful result"); *Fuller v. Berger*, 120 F. 274, 275 (7th Cir. 1903) (test for utility is whether invention "is capable of serving any beneficial end").

Juicy Whip, Inc. v. Orange Bang Inc., 185 F.3d 1364, 1366, 51 USPQ2d 1700, 1702 (Fed. Cir. 1999). Clearly the claimed invention is *capable* of providing some identifiable benefit, *i.e.*, antibodies raised against EBI-2 G-protein coupled receptor are *capable* of being useful as therapeutics for myocardial infarction. In fact, as shown previously in Applicants' Amendment and Reply Under 37 C.F.R. § 1.111 filed May 6, 2003, antagonists to the EBI-2 receptor, such as the claimed antibodies, can be used as suggested in the specification to treat myocardial infarction.

B. The Specification Discloses at Least One Specific Utility

The Examiner asserted that

no disclosure is provided within the instant specification as to any specific biological function of the polypeptide having SEQ ID NO:2 or any specific disease where the claimed invention could be used. Speculating a function of a protein merely based on homology is not predictive. Any benefit to the public is speculative at best.

(Office Action, page 5.)

The assertion that the specification fails to disclose a specific biological function or any specific disease state where the claimed invention could be used is simply incorrect. Regarding the specificity of an asserted use, the Utility Guidelines define "specific utility" as a utility that

is *specific* to the subject matter claimed. This contrasts with a *general* utility that would be applicable to the broad class of the invention. . . . For example, indicating that a compound maybe useful in treating unspecified disorders, or that the compound has "useful biological" properties, would not be sufficient to define a specific utility for the compound.

MPEP § 2107.01 at 2100-32.

Applicants assert that the specification does not assert that the claimed invention "maybe useful in treating unspecified disorders." The specification indicates that "GPCR molecules and their associated G-proteins have been implicated in . . . adenylyl cyclase signal channels" (Specification, ¶ [0035].) Further, in view of the expression sites of EBI-2 G-protein coupled receptor, its amino acid sequence as well as the knowledge of other G-protein coupled receptors, among other things, it was asserted in the specification that antagonists of the EBI-2 G-protein coupled receptor can be used to treat, *inter alia*, myocardial infarction. The specification also discloses examples of inhibitors of the EBI-2 G-protein coupled receptor which include antibodies, small molecules and soluble forms of the receptor. (See Specification, ¶¶ [0097] and [0099].) The use of antibodies against EBI-2 G-protein coupled receptor molecules to treat, for example, myocardial infarction is a specific use that is not generally applicable to all G-protein coupled receptors, much less to all proteins. Thus, Applicants submit that the specification discloses at least one specific utility for the claimed invention.

In addition, the Examiner appears to take into consideration only the homology information provided for EBI-2 G-protein coupled receptor. In response, Applicants note that, according to the USPTO, "there is no per se rule regarding homology, and each application must be judged on its own merits." (Federal Register, Vol. 66, No. 4, 1096 (Friday, January 5, 2001).) As appreciated by those of ordinary skill in the art, in many instances sequence similarity is predictive of protein function. Nevertheless, it is evident from the record that Applicants have provided *more* than just homology information in support of the assertions of utility for the claimed invention.

Moreover, Applicants note that "Office personnel should be careful . . . not to label certain types of inventions as . . . 'speculative' as such labels do not provide the correct focus for the evaluation of an assertion of utility." MPEP §2107.02 at 2100-40. Contrary to what the Examiner suggests, Applicants need not confirm any asserted utility. An assertion of utility need only be "reasonably predictive" (as opposed to "reasonably confirmed"); it need not be a "statistical certainty." *See, e.g., Rey-Bellet v. Englehardt*, 493 F.2d 1380 (CPPA 1974); MPEP § 2107.01; *Nelson v. Bowler*, 626 F.2d 853, 856-57 (CPPA 1980). The standard is whether one skilled in the art would reasonably conclude that the asserted utility is more likely than not true. Applicants' assertion of utility is more than mere speculation. Based on the evidence on record, Applicants assert that this standard has been satisfied.

C. There is a Nexus Between the Diseases Discussed in the Specification and the Claimed Subject Matter.

It is also the Examiner's position that

[t]he claimed antibody is directed against the protein of SEQ ID NO:2, which is an orphan receptor without a known ligand and whose biological function is unknown. The specification does not disclose a nexus between the orphan receptor and heart disease or any other pathological condition for that matter. The fact that the protein is an orphan receptor indicates that its role in any putative disease progress has yet to be elucidated. In the absence of any data as to the receptor's biological function, there is no basis upon which to base a specific or substantial utility for the claimed antibody.

(Office Action, page 5.)

The Examiner appears to require that Applicants demonstrate a reasonable linkage or nexus between the claimed antibodies against EBI-2 G-protein coupled

receptors, and *each* specific and substantial utility disclosed in the specification, or at least the generic "category" of utilities disclosed. As Applicants understand the Examiner's position, the skilled artisan should be able to reasonably confirm a specific and substantial utility without further research.

While not agreeing with the Examiner's position, Applicants nonetheless point out that a reasonable linkage or nexus between the claimed antibodies against EBI-2 G-protein coupled receptors, and the general category of specific and substantial utilities disclosed in the specification does, indeed exist. Applicants identified the EBI-2 G-protein coupled receptors in cDNA libraries from umbilical vein endothelial cells (vascular tissue), neutrophil leukocyte cells (myeloid tissue, derived from the same progenitor cells as lymphoid tissue) and corpus callosum cells (brain or nervous tissue). (See Specification, ¶ [0032].) Given the expression of EBI-2 mRNA in these tissues, one of ordinary skill in the art would logically expect functions and utilities related to vascular, cardiac or nervous system disorders.

In addition, the G protein-coupled receptor encoded by SEQ ID NO:1 is closely related to certain G protein-coupled receptors known to be induced upon Epstein-Barr virus (EBV) infection. (See Specification, ¶ [0003].) These related EBI receptors are primarily expressed in lymphoid tissues and are related to thrombin receptors. See Birkenbach *et al.*, *J. Virol.* 67:2209-2220 (1993)(Exhibit A). Further, EBV and related gamma-herpesvirus infections are often associated with vascular and cardiac diseases. See, e.g., Kikuta *et al.*, *J. Pediatr.* 123:90-92 (1993)(Exhibit B); and Tyson *et al.*, *South. Med. J.* 82:1184-1187 (1989)(Exhibit C).

Based on the relationship to EBI receptors, one of ordinary skill in the art would again logically expect functions and utilities related to cardiac disorders. As such, there is clearly a direct nexus between the G-protein coupled receptor (EBI-2) and heart disease, specifically myocardial infarction. Thus, the claimed antibodies certainly provide an identifiable benefit which is easily linked to diseases and disorders disclosed in the specification.

Indeed, the reasonableness of the asserted utility was confirmed by Hollopeter *et al.*, *Nature* 409(6817):202-7 (2001), which was submitted with the Amendment and Reply Under 37 C.F.R. § 1.111 filed May 6, 2003. Hollopeter *et al.* demonstrate that the EBI-2 receptor of the present invention is primarily expressed in platelets (myeloid lineage) and brain (*see* Fig. 4), and that differential EBI-2 expression may indicate a perturbation in platelet aggregation, which can lead to cardiovascular diseases, including myocardial infarction. Thus, this publication confirms a reasonable nexus between EBI-2 as disclosed in the present application, and the treatment and/or detection of heart disease.

D. The Specification Discloses at Least One Specific and Substantial Utility

The Examiner further alleged that the claimed invention does not have at least one asserted, specific utility which is substantial. (*See* Office Action, page 6.) In particular, the Examiner indicated that

Applicants assert that the specification [0035, page 8, 0097, page 23, 0098, page 24, 0099, page 24], discloses a role of the claimed invention in potential diverse therapeutic and diagnostic applications including mental disorders, cancer, migraine, eating disorders, asthma, heart

disease, psychoses, restenosis, Alzheimer's disease, Parkinson's disease, atherosclerosis and a number of others. The applicants' disclosure provides a large list of diseases allegedly associated with the polypeptide of SEQ ID NO:2, but fails to disclose the specific role of the disclosed protein in any of these diseases.

(Office Action, page 6.)

Applicants respectfully re-emphasize that the specification discloses at least one specific and substantial utility for the claimed invention. A substantial utility is one that defines a "real world" use. In addition, "any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a 'substantial' utility." MPEP § 2107.01 at 2100-33. Further, a therapeutic method of treating a known disease defines a "real world" context of use. *Id.* at 2100-32. The use of antibodies which bind to the EBI-2 G protein coupled receptor molecules to treat a known disease, for example, myocardial infarction, is a substantial utility as it provides a benefit to the public.

Applicants further note that they need only provide *one* credible assertion of specific and substantial utility for the claimed invention to satisfy the utility requirement of § 101. *See* MPEP § 2107.02 at 2100-37. Additional statements of utility, even if not "credible," do not render the claimed invention lacking in utility. *See id.*; *see also Raytheon v. Roper*, 220 U.S.P.Q. 592, 598 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 835 (1984) ("When a properly claimed invention meets at least one stated objective, utility under 35 U.S.C. § 101 is clearly shown.").

The specification discloses that G-protein coupled receptor molecules, such as EBI-2 G protein coupled receptor, and their associated G-proteins have been implicated

in adenylyl cyclase signal channels. (*See* specification, ¶ [0035].) Further, the specification discloses that antagonists to the EBI-2 G protein coupled receptor, such as the claimed monoclonal antibodies, would be useful for the treatment of myocardial infarction. (*See, e.g.,* specification, paragraphs [0097] and [0099].)

Consistent with the specification, Hollopeter *et al.* (previously submitted) and Dorsam *et al.*, *The Journal of Clinical Investigation* 113:340-345 (2004) (Exhibit D) disclose that the EBI-2 G protein coupled receptor is coupled to the inhibition of adenylyl cyclase which leads to platelet aggregation. Also consistent with the specification, the authors of these publications indicate that perturbations in this EBI-2 receptor-mediated activity can lead to cardiovascular diseases, including myocardial infarction, and that antagonists to the EBI-2 receptor would be useful to treat myocardial infarction.

The references of record confirm that antagonists to the EBI-2 receptor, such as the claimed antibodies, can be used as suggested in the specification, *i.e.* to treat myocardial infarction. As such, Applicants have disclosed a "therapeutic method of treating a known disease." Since one skilled in the art can use the claimed discovery in a manner which provides some immediate benefit to the public, Applicants submit that the disclosed utilities are substantial utilities as they provide real world value.

E. The Use of Post-filing Date References to Substantiate Assertions of Utility is Proper

The Examiner further asserted that:

Applicants point to the findings of Wang, Hollopeter and Chattaraj in order to establish a utility of the instant invention. However, Applicants have failed to disclose an

asserted, substantial and specific utility of their invention in the specification at the time [of] filing the instant application.

(Office Action, page 7.)

Applicants assert that they are *not* using post-filing date art to establish utility for the claimed invention. Rather, Applicants have brought post-filing date art to the attention of the Examiner to *substantiate* one of the substantial utilities specifically asserted in the specification. Applicants note that both the PTO and Federal Circuit indicate that post-filing art can be used to *substantiate* a specific and substantial utility asserted in the specification. In *In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995), the Federal Circuit expressly recognized the use of post-filing date declarations and references to substantiate any doubts as to an asserted utility of an invention so long as the reference "pertains to the accuracy of a statement already in the specification. . . . It does not render an insufficient disclosure enabling, but instead goes to prove that the disclosure was in fact enabling when filed (i.e., demonstrated utility)." *In re Brana*, 51 F.3d 1560, 1567 n.19 (Fed. Cir. 1995).

In addition, the MPEP § 2107.02 states that "[i]n appropriate situations the Office may require an applicant to substantiate an asserted utility for a claimed invention. See *In re Pottier*, 376 F.2d 328, 330, 153 USPQ 407, 408 (CCPA 1976)." Furthermore:

If the applicant responds to the *prima facie* [35 U.S.C. § 101] rejection, Office personnel should review the original disclosure, any evidence relied upon in establishing the *prima facie* showing, any claim amendments, and *any new reasoning or evidence provided by the applicant in support of an asserted specific and substantial credible utility.*

MPEP § 2107.02 at 2100-43 (*emphasis added*). Thus, Applicants may provide "any new reasoning or evidence" such as post filing art references to substantiate an asserted utility.

Thus, Applicants submit that the use of post-filing date references and the data provided therein to corroborate assertions of utility with respect to the instant invention is proper. Applicants respectfully assert, therefore, that the remarks and arguments relating to post-filing date references in response to the utility rejection made in the Amendment and Reply Under 37 C.F.R. § 1.111 filed May 6, 2003, are fully applicable and are incorporated by reference herein.

The Examiner also cited *In re Kirk* for the proposition that Applicants cannot

satisfy the requirements of the statutes by indicating the usefulness of a claimed compound in terms of possible use so general as to be meaningless and then, after his research or that of his competitors has definitely ascertained an actual use for the compound, adducing evidence intended to show that a particular specific use would have been obvious

(Office Action, page 7.)

As discussed *supra*, Applicants can provide post-filing date references as evidence of corroboration of an asserted utility. Further, Applicants acknowledge that "nebulous expressions, such as 'biological activity' or 'biological properties,' disclosed in a specification convey little explicit indication regarding the utility of a compound." *Cross v. Iizuka*, 753 F.2d 1040, 1048 (Fed. Cir. 1985) (quoting *In re Kirk*, 376 F.2d 936, 941 (CCPA 1967). However, unlike the applicants in *In re Kirk* who asserted in the specification that the claimed steroid compounds possess "high biological activity,"

Applicants have not defined the utility of the presently claimed invention using such general and meaningless expressions. Rather, the specification discloses the use of the claimed EBI-2 G-protein coupled receptor antibodies to treat, for example, myocardial infarction. Such an expression clearly conveys an explicit indication regarding the utility of the claimed compounds.

Applicants have asserted that the claimed antibodies can be used, for example, for the diagnosis and/or treatment of heart diseases such as myocardial infarction. In view of the facts and relationships set out above, Applicants submit that this assertion is not only specific and substantial, but credible as well, *i.e.*, the assertion is *at least believable* to, and would not be considered *false* by, a person of ordinary skill in the art. *See* MPEP § 2107.02 at 2100-40. Since the presently claimed invention possesses a credible, specific and substantial utility that constitutes a patentable utility under 35 U.S.C. § 101, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of the claims under 35 U.S.C. § 101.

V. Rejections under 35 U.S.C. § 112, First Paragraph

A. Enablement

The Examiner maintained the rejection of claims 23-74 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement. (*See* Office Action, page 7.) In particular, it is the Examiner's position that "[s]ince the claimed invention is not supported by either a credible, specific and substantial asserted utility or a well

established utility for the reason set forth above, one skilled in the art clearly would not know how to use the claimed invention." (*Id.*)

For the reasons discussed above in reply to the rejection under 35 U.S.C. § 101, Applicants assert that the claimed invention complies with current case law and is supported by a specific, substantial and credible utility as well. The Examiner "should not impose a 35 U.S.C. 112, first paragraph, rejection grounded on a 'lack of utility' basis unless a 35 U.S.C. 101 rejection is proper." MPEP § 2107.01 at 2100-36. Therefore, since the claimed invention complies with the utility requirement of 35 U.S.C. § 101, the rejection under 35 U.S.C. § 112, first paragraph, based on the alleged lack of utility of the claimed invention, should be withdrawn.

The Examiner has withdrawn the rejection of claims 39, 50, 65 and 74 under 35 U.S.C. § 112, first paragraph, in view of Applicants' arguments. (*See* Office Action, page 8.) Applicants thank the Examiner for the withdrawal of these rejections.

B. Written Description

The Examiner has withdrawn the rejection of claims 39, 50, 65 and 74 under 35 U.S.C. § 112, first paragraph, in view of Applicants' arguments and/or amendments. (*See* Office Action, page 8.) Applicants thank the Examiner for the withdrawal of these rejections.

C. Deposit Rules

The Examiner has withdrawn the rejection of claims 51-74 under 35 U.S.C. § 112, first paragraph, in view of Applicants' Statement filed May 6, 2003. (*See* Office Action, page 9.) Applicants thank the Examiner for the withdrawal of these rejections.

VI. New Grounds of Rejection

A. Rejections under 35 U.S.C. § 112, Second Paragraph

The Examiner rejected claims 29 and 30 under 35 U.S.C. § 112, second paragraph, as allegedly "being vague and indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention." (Office Action, page 9.) Specifically, the Examiner asserted that "[i]t is not clear how 'hybridoma technique' differ[s] from the human B-cell hybridoma technique and the EBV-hybridoma technique. Further, it is unclear which hybridoma technique claim 30 refers back to." (*Id.*)

Applicants respectfully disagree with the Examiner. However, solely to advance prosecution, and not in acquiescence to the Examiner's rejection, Applicants have amended claims 29, 32, 33, 55, 58 and 59 to further clarify methods by which the claimed antibodies can be produced. Applicants submit that these claims clearly particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Thus, Applicants respectfully request that the Examiner reconsider and withdraw this rejection.

B. Rejections under 35 U.S.C. § 112, First Paragraph

1. Written Description

The Examiner rejected claim 36 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. (*See* Office Action, page 9.) Applicants believe that claim 36 contains subject matter that was described in the specification in such a way as to reasonably convey to one skilled in the relevant art

that the inventors, at the time the application was filed, had possession of the claimed invention. Furthermore, methods of producing antibodies using transgenic mice, *e.g.* mice engineered to produce human antibodies, were well known in the art as of the earliest effective filing date of the instant application. However, solely to advance prosecution, and not in acquiescence to the Examiner's rejection, Applicants have canceled claim 36, thereby rendering the rejection moot. Thus, Applicants respectfully request that the Examiner reconsider and withdraw this rejection.

2. Enablement

The Examiner rejected claim 36 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. (*See* Office Action, page 12.) Applicants believe that claim 36 contains subject matter that was described in the specification in such a way as to enable one skilled in the relevant art to make and use the claimed invention without undue experimentation. However, solely to advance prosecution, and not in acquiescence to the Examiner's rejection, Applicants have canceled claim 36, thereby rendering the rejection moot. Thus, Applicants respectfully request that the Examiner reconsider and withdraw this rejection.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the

Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Epstein-Barr Virus-Induced Genes: First Lymphocyte-Specific G Protein-Coupled Peptide Receptors

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Since Epstein-Barr virus (EBV) infection of Burkitt's lymphoma (BL) cells in vitro reproduces many of the activation effects of EBV infection of primary B lymphocytes, mRNAs induced in BL cells have been cloned and identified by subtractive hybridization. Nine genes encode RNAs which are 4- to >100-fold more abundant after EBV infection. Two of these, the genes for CD21 and vimentin, were previously known to be induced by EBV infection. Five others, the genes for cathepsin H, annexin VI (p68), serglycin proteoglycan core protein, CD44, and the myristylated alanine-rich protein kinase C substrate (MARCKS), are genes which were not previously known to be induced by EBV infection. Two novel genes, EBV-induced genes 1 and 2 (EBI 1 and EBI 2, respectively) can be predicted from their cDNA sequences to encode G protein-coupled peptide receptors. EBI 1 is expressed exclusively in B- and T-lymphocyte cell lines and in lymphoid tissues and is highly homologous to the interleukin 8 receptors. EBI 2 is most closely related to the thrombin receptor. EBI 2 is expressed in B-lymphocyte cell lines and in lymphoid tissues but not in T-lymphocyte cell lines or peripheral blood T lymphocytes. EBI 2 is also expressed at lower levels in a promyelocytic and a histiocytic cell line and in pulmonary tissue. These predicted G protein-coupled peptide receptors are more likely to be mediators of EBV effects on B lymphocytes or of normal lymphocyte functions than are genes previously known to be up-regulated by EBV infection.

Epstein-Barr Virus (EBV) is the cause of infectious mononucleosis, a benign proliferation of infected B lymphocytes (36), and can also cause acute and rapidly progressive B-lymphoproliferative disease in severely immunocompromised patients or in experimental infection of tamarins (56). Infection of human B lymphocytes in vitro results in expression of six virus-encoded nuclear proteins (EBNAs) and two virus-encoded membrane proteins (LMPs) (44) and in substantially altered cell growth (61, 62). EBV-infected B lymphocytes recapitulate features of antigen stimulation in enlarging, increasing RNA synthesis, expressing activation antigens and adhesion molecules, differentiating toward immunoglobulin (Ig) secretion, and proliferating (10, 26, 31, 61, 79, 80). Unlike antigen-stimulated B lymphocytes, EBV-infected B lymphocytes continue to proliferate in vitro as immortalized lymphoblastoid cell lines (62).

EBV effects on lymphocytes have been studied by comparing the properties of EBV-negative [EBV(-)] Burkitt's lymphoma (BL) cell lines and EBV-positive [EBV(+)] derivatives, infected by EBV, in vitro (12, 21, 61, 66-68). EBV(-) BL cells resemble proliferating centroblasts of germinal centers, characteristically expressing CD10, CD20, CD77 (BLA), class II antigen, and the carbohydrate recognized by peanut agglutinin (12, 21, 23, 28-30, 66, 67). Both EBV(-) BL cells and centroblasts lack surface IgD and antigens associated with early phases of mitogen stimulation in vitro, including CD23, CD39, and CD30. In general, EBV(+) BL cells closely resemble EBV-infected primary B lymphocytes in not expressing CD10 or CD77 and in expressing early activation and differentiation markers, vimen-

tin, Bac-1, Bcl-2, surface IgD, and CD44 (12, 21, 23, 29, 35, 66, 68, 70, 76, 84-86).

Experiments with single-gene transfer into EBV(-) B-lymphoma cells or with specifically mutated EBV recombinants reveal that EBNA 2, LMP 1, and EBNA 3C are essential for lymphocyte growth transformation and alter cellular or viral gene expression. Expression of EBNA 2 alone in EBV(-) BL cell lines results in enhanced transcription of CD23, CD21 (17, 85, 86), and *c-fgr* (46). EBNA 2 also transactivates the LMP promoters (22, 87). Analysis of a series of EBNA 2 mutants indicates that the ability of EBNA 2 to transactivate gene expression is tightly linked to its essential role in cell growth transformation (16). LMP 1 is also critical to the effects of EBV on cell growth. LMP 1 transforms immortalized rodent fibroblasts (5, 82, 83) and induces vimentin, Bcl-2, and many of the activation markers and adhesion molecules that EBV induces in BL cells or primary B lymphocytes (8, 35, 84, 85). In EBV(-) BL cells, EBNA 3c induces higher-level expression of CD21 (85).

Since altered B-lymphocyte gene expression is a central theme in EBV-induced changes in B-lymphocyte growth, a more complete description of the repertoire of EBV-induced genes would be advantageous before the investigation of specific genes for their role as mediators of EBV effects on cell growth. Genes induced by EBV could also be of importance in B-lymphocyte immunology because of the similar activating and differentiating effects of EBV and antigen. Previously, recognition of such genes has been based largely on increased expression of lymphocyte surface markers (12, 85, 86), defined by monoclonal antibodies derived against EBV- or antigen-activated B lymphocytes. Few of these surface markers are likely candidates for important effectors of EBV- or antigen-induced alterations in

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lymphocyte growth. The experiments described here use subtracted hybridization to identify cDNA clones of RNAs which are more abundant in an in vitro-infected EBV(+) BL cell than in the noninfected EBV(-) control BL cell.

MATERIALS AND METHODS

Cells and cell lines. BL41 and BL30 are EBV(-) BL cell lines. The BL41/B95-8 and BL41/P3HR1 cell lines were derived by infecting BL41 with the transforming EBV strain B95-8 or the nontransforming strain P3HR1, respectively (12, 23). IB4 is a latently infected B lymphoblastoid cell line established by infection of B lymphocytes with EBV (B95-8) in vitro. RHEK-1 (a generous gift from Jong Rhim, National Cancer Institute) is a human keratinocyte line derived by infection of primary foreskin epithelial cells with an adenovirus 12-simian virus 40 hybrid. K562 is a Philadelphia chromosome-positive human chronic myeloid leukemia cell line. U937 is a histiocytic lymphoma cell line with monocytic features. HL60 is a promyelocytic leukemia line. HSB-2 and Jurkat are human T lymphoblastic leukemia cell lines. TK143 was derived from a human osteosarcoma.

Human peripheral blood mononuclear cells (PBMCs) were purified from peripheral blood by centrifugation on a Ficoll cushion (Ficoll-Hypaque; Pharmacia, Vineland, N.J.). Cells were resuspended at 10^6 cells per ml in RPMI 1640 medium supplemented with 20% fetal bovine serum and were divided into parallel cultures grown for 72 h with or without 2.5 μ g of pokeweed mitogen (Sigma, St. Louis, Mo.) per ml. T cells were isolated from purified PBMCs by rosetting overnight with aminoethylisothiuronium bromide (AET)-treated sheep erythrocytes at 4°C followed by centrifugation over Ficoll. Pelleted erythrocytes were lysed with ammonium chloride. The remaining T cells were resuspended at 10^6 cells per ml in RPMI 1640 medium with 20% fetal bovine serum. Phytohemagglutinin (Sigma) was added to a final concentration of 1.0 μ g/ml. Cells were cultured for 72 h and harvested for extraction of total cellular RNA.

RNA preparation and analysis. Cytoplasmic RNA was isolated from exponentially growing cells by a modification of the acid phenol-guanidinium isothiocyanate extraction procedure followed by reprecipitation in guanidinium hydrochloride-ethanol. Total cellular RNA was extracted from 0.2- to 2-g samples of human spleen and tonsil obtained from surgical specimens and from human bone marrow obtained postmortem. Tissues were homogenized in acid phenol-guanidinium isothiocyanate by using a rotary tissue homogenizer, extracted, and precipitated. After dissolution in guanidinium hydrochloride and reprecipitation with ethanol, human tissue RNA samples were resuspended in H₂O and precipitated by addition of an equal volume of 8 M LiCl. The polyadenylated fractions of BL41 or BL41/B95-8 RNA were purified by two successive cycles of chromatography on oligodeoxythymidylate cellulose. Polyadenylated IB4 RNA was purified by a single round of oligodeoxythymidylate selection. RNA samples (12 μ g per lane) were size fractionated on 0.66 M formaldehyde-1% agarose gels and transferred to charged nylon membranes (GeneScreen Plus; New England Nuclear, Billerica, Mass.) for subsequent hybridization analysis. To examine gene expression in other human tissues, we used a commercially prepared blot containing 2 μ g each of polyadenylated heart, brain, placenta, lung, liver, kidney, skeletal muscle, and pancreas RNA (Multiple Tissue Northern; Clontech, Palo Alto, Calif.).

Probes were prepared from cloned cDNA inserts by using random hexamer primers and [³²P]dCTP. The β -actin probe

was generated by using a previously described 1.4-kb cDNA (8). The glyceraldehyde phosphate dehydrogenase probe was prepared from a commercially obtained DNA fragment (Clontech). Filters were hybridized for 18 to 24 h at 47°C in a hybridization buffer consisting of 50% formamide, 6 \times SSPE (20 \times SSPE is 3.0 M NaCl, 200 mM NaPO₄ [pH 7.4] and 20 mM EDTA), 1% sodium dodecyl sulfate (SDS), 1 \times Denhardt's solution (100 \times Denhardt's solution is 2% bovine serum albumin, 2% polyvinylpyrrolidone, and 2% Ficoll), and 100 μ g of sheared single-stranded herring testis DNA per ml. The filters were washed as specified by the manufacturer, with high stringency washes performed at 67 to 70°C in 1% SDS-0.2 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and exposed to preflashed film (X-Omat AR; Kodak, Rochester, N.Y.) at -80°C for 2 h to 10 days. Autoradiographic signal intensities were quantitated by densitometric scanning with a Beckman DU-8 spectrophotometer equipped with a slab gel Compuset module. Induction factors were calculated for each probe as signal intensity ratios for EBV(+) versus EBV(-) cells, divided by the ratio of β -actin signal intensities.

cDNA library preparation. First-strand cDNA was prepared from 5 μ g of polyadenylated BL41/B95-8 RNA by using Moloney murine leukemia virus reverse transcriptase (SuperScript; Bethesda Research Laboratories, Gaithersburg, Md.) and oligodeoxythymidylate primers in a 100- μ l reaction. Second-strand cDNA was synthesized by using *Escherichia coli* DNA polymerase I and RNase H. The double-stranded cDNA was blunt ended with T4 DNA polymerase and *Eco*RI methylated. After ligation of *Eco*RI linkers, the cDNA was digested with *Eco*RI and size fractionated by gel filtration chromatography on Sepharose CL-4B. The purified cDNA was ligated to phosphorylated lambda gt10 arms (Promega, Madison, Wis.) and packaged (Gigapack Gold; Stratagene, La Jolla, Calif.).

Subtractive probe preparation. Radiolabeled cDNA was prepared from 6 μ g of polyadenylated BL41 or BL41/B95-8 RNA in a 200- μ l reaction containing 50 μ g of random DNA hexamers per ml; 0.5 mM each dATP, dGTP, and dTTP; 25 μ M unlabeled dCTP; 1.0 mCi of [³²P]dCTP (800 Ci/mmol; New England Nuclear); and 2,000 U of recombinant Moloney murine leukemia virus reverse transcriptase. Reactions were carried out at 42°C for 1 h. After precipitation, reaction products were resuspended in 0.1 M NaOH and incubated for 20 min at 65°C to hydrolyze RNA templates. Probes were neutralized with 0.1 M acetic acid and size fractionated on Sephadex G-50. Biotinylated RNA was prepared from polyadenylated BL41 RNA by using a photoactivatable azido-aryl biotin reagent (Photoprobe Biotin; Vector Laboratories, Burlingame, Calif.) as specified by the manufacturer. Probe fractions were combined with 48 μ g (for BL41/B95-8 probe) or 12 μ g (for BL41 probe) of biotinylated BL41 RNA and precipitated with ethanol. BL41/B95-8 probes were hybridized with an eightfold excess (2 mg/ml) of biotinylated BL41 RNA, whereas BL41 control probes were hybridized with a twofold excess (0.5 μ g/ml) of biotinylated BL41 RNA. Hybridizations and subtractions were performed with the Subtractor kit (Invitrogen, San Diego, Calif.) as specified by the manufacturer instructions. The precipitated cDNA-RNA mixtures were resuspended in 10 to 20 μ l of H₂O and heated to 100°C for 1 min. An equal volume of 2 \times hybridization buffer (Invitrogen) was added, and the mixture was incubated at 65°C for 20 to 24 h. Following addition of an equal volume of HEPES buffer (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.5], 1 mM EDTA), 20 μ g of streptavidin was added and the mixture

was incubated on ice for 10 min. Biotinylated RNA and RNA-cDNA duplexes, complexed with avidin, were removed by repeated phenol-chloroform extractions. The single-stranded, subtracted BL41 cDNA probe which remained in the aqueous phase was used directly for in situ filter hybridizations. Aqueous-phase BL41/B95-8 cDNA probe was precipitated with ethanol and subjected to a second round of subtraction under identical conditions prior to use in filter hybridizations. Duplicate filters were made from 145-mm plates containing 6,000 recombinant bacteriophage and were hybridized in parallel to equal amounts of BL41/B95-8 or BL41 subtracted probes. The filters were hybridized at 48°C for 48 to 72 h in a buffer consisting of 50% formamide, 6× SSPE, 1% SDS, 10% dextran sulfate, 2× Denhardt's solution, 100 µg of sheared single-stranded herring testis DNA per ml, and 10 µg of poly(rA-rU) (Sigma) per ml. They were then washed at 72°C in 0.2× SSC and exposed to preflashed film (Kodak X-Omat AR) for 3 to 7 days. Differentially expressed genes were identified by overlaying films from corresponding filters. Clones selected on primary screening were rescreened once at low density to verify differential expression and for plaque purification.

Analysis of clones. DNA was extracted from bulk liquid cultures of purified lambda gt10 clones and digested with *Eco*RI. cDNA inserts were purified by agarose gel electrophoresis and subcloned into pBluescript(+). Nucleotide sequences were determined and were compared by the BLAST algorithm (3) with known sequences resident in the National Center for Biotechnology Information data bases by using the Experimental GENINFO(R) BLAST Network Service, accessed through the Molecular Biology Computer Research Resource of the Dana-Farber Cancer Institute. Multiple sequence alignments were performed by the method of Higgins and Sharp (37), using the CLUSTAL program (PCGene; IntelliGenetics, Mountain View, Calif.) with open gap and unit gap costs of 10.

Nucleotide sequence accession numbers. GenBank accession numbers are L08176 for EBI 1 and L08177 for EBI 2.

RESULTS

Identification of cDNA clones of EBV-induced RNAs by subtracted-probe hybridization. cDNA clones of RNA from an in vitro EBV-infected BL cell line, BL41/B95-8 [EBV(+) BL41], were differentially screened with an EBV(+) BL41 cDNA probe from which sequences complementary to EBV(-) BL41 cell RNA had been specifically removed and with an EBV(-) BL41 control cDNA probe. Sequences complementary to EBV(-) BL41 RNA were removed from the EBV(+) BL41 RNA cDNA probes by two subtractions with an eightfold excess of biotinylated EBV(-) BL41 RNA. Overall, 85 to 95% of the labeled EBV(+) BL41 probe was removed by the two subtractions. EBV(-) BL41 cDNA control probe was subtracted only once; this removed 60 to 85% of the probe, thereby reducing hybridization to plaques containing cDNAs from abundant RNAs so that hybridization to cDNAs from less abundant BL41 RNAs was evident.

Seventy-five phage cDNA clones differentially hybridized to the EBV(+) BL41 probe on the first screen of 75,000 recombinant phage. Twenty-five clones were consistently positive on rescreening. The 18 clones which demonstrated the greatest reactivity with the EBV(+) versus the EBV(-) BL41 cDNA probes were selected for nucleotide sequencing and RNA blot hybridization.

TABLE 1. Summary of EBV-induced RNA-DNA clones

Clone	Gene	cDNA size (kb)	RNA size (kb)	Induction ^a
1.1	CD44	1.3	1.6, 2.2, 5.0	>100×
3.3, 7.3	CD21	2.1, 1.8	4.8	
6.5	MARCKS	2.6	2.9	30×
8.2	Cathepsin H	1.5	1.7	6×
10.4, 11.4	Serglycin	1.1, 1.1	1.4	3.5×
12.3	Annexin VI	2.3	3.0	5×
12.5, 13.0	Vimentin	1.0, 1.8	2.0	
6.4	EBI 1	1.2 (2.14) ^b	2.4	21×
3.2	EBI 2	1.64	1.9	>200×
	β-Actin		2.2	3×

^a Induction levels were calculated as ratio of signal intensities (BL41/B95-8 to BL41) for individual probes, divided by the ratio of signal intensities for the β-actin probe.

^b The 1.2-kb EBI 1 clone identified on the initial screen was incomplete. Rescreening of the cDNA library resulted in isolation of several additional full-length clones, the largest of which was 2.14 kb.

^c Induction of β-actin RNA was calculated as the ratio of actin signal intensities (BL41/B95-8 to BL41) divided by the ratio of signal intensities for glyceraldehyde phosphate dehydrogenase probe.

Nucleotide sequences of EBV-induced cDNAs. The first 12 clones are described in Table 1. Ten clones matched seven previously characterized genes: two independent clones each of the genes for complement receptor type 2 (CD21), the serglycin proteoglycan core protein, and vimentin; and one clone each of the genes for cathepsin H, annexin VI (p68), the myristylated alanine-rich protein kinase C substrate (MARCKS), and the lymphocyte hyaluronic acid receptor (CD44). The 2.6-kb MARCKS cDNA precisely matched the previous 1.58-kb human MARCKS cDNA clone (32) at its 5' end (7a). The 3' untranslated region of the new clone is highly homologous to bovine MARCKS cDNA (75).

The two remaining clones are from novel RNAs, EBV-induced genes 1 (EBI 1) and 2 (EBI 2), whose nucleotide sequences can be predicted to encode G protein-coupled peptide receptors. The complete nucleotide and deduced amino acid sequences of the EBI 1 and EBI 2 cDNAs are shown in Fig. 1A B, respectively. Because the first EBI 1 cDNA was 1.2 kb, significantly shorter than the 2.4-kb RNA, 20 other cDNA clones were obtained by using the initial cDNA as a probe. The largest clone is 2,153 nucleotides (nt) and has a 1,134-nt open reading frame (Fig. 1A). This clone is probably nearly full length, since it is close to the expected size, considering that it has only a short poly(A) tail. Translation is likely to initiate from either of two AUGs, at nt 64 to 66 or nt 82 to 84, the first of which conforms to a consensus translational initiation sequence (49). An in-frame stop codon at nt 10 to 12 is consistent with translational initiation at nt 64 to 66. The polypeptide encoded by the sequence beginning at nt 64 has a predicted molecular mass of 42.7 kDa and includes eight hydrophobic domains likely to mediate membrane insertion. The first hydrophobic domain begins at the amino terminus and ends at a predicted signal peptidase cleavage site. The seven remaining hydrophobic domains are characteristic of the G protein-coupled receptor family. A potential asparagine-linked glycosylation site is encoded as part of the extracellular amino-terminal segment and as part of the third extracellular loop.

Since the initial EBI 2 cDNA was 1,643 nt and approximated the size expected from a 1.9-kb polyadenylated RNA, further cDNA clones were not obtained. The EBI 2 cDNA contains a 1,083-nt open reading frame with two methionine

A

GGAATTCCTAGTGGGAGGCGGGCACAGCCTTCCTGTGTGTTTACCGCCAGAGAGCGTCATGGACCTGGGAAACCAATGAAAAGC 90
 *** METAspLeuGlyLysProMetLysSer
 GTGCTGGTGGTGGCTCTCTTGTGCTTTTCCAGGTATGCTGTGTCAAGATGAGGTACCGGACGATTACATCGGAGACAACACCACTG 180
 ValLeuValValAlaLeuLeuValIlePheGlnValCysLeuCysGlnAspGluValThrAspAspTyrIleGlyAspAsnThrThrVal 39
 CHO#####
 GACTACACTTGTTCGAGTCTTTGTGCTCCAAGAAGGACGTCCGGAACCTTAAAGCGTGGTTCCTCGCTATCATGTACTCCATCAITTTGT 270
 AspTyrThrLeuPheGluSerLeuCysSerLysLysAspValArgAsnPheLysAlaTrpPheLeuProIleMetTyrSerIleIleCys 69
 TTCGTGGGCTACTGGGCAATGGGCTGGTGTGACCTATATCTATTCAAGAGGCTCAAGACCATGACCGATACCTACCTGCTCAAC 360
 PheValGlyLeuLeuGlyAsnGlyLeuValValLeuThrTyrIleTyrPheLysArgLeuLysThrMetThrAspThrTyrLeuLeuAsn 99
 CTGGCGGTGGCAGACATCCTCTCTCTGACCTTCCCTTCTGGGCTACAGCGGGCCCAAGTCTGGGTCTTGGGTGTCCACTTTTGC 450
 LeuAlaValAlaAspIleLeuPheLeuLeuProPheTrpAlaTyrSerAlaAlaLysSerTrpValPheGlyValHisPheCys 129
 AAGCTCATCTTTGCCATCTACAAGATGAGCTTCTTCACTGGCATGCTCTAGTTCTTTGTCATCAGCATTGACCGCTACGTGGCCATCGTC 540
 LysLeuIlePheAlaIleTyrLysMetSerPhePheSerGlyMetLeuLeuLeuCysIleSerIleAspArgTyrValAlaIleVal 159

 CAGGCTGTCTCAGCTCACCGCCACCGTGGCGGCTCTCTCATCAGCAAGCTGTCTGTGGGACGGCCATAGTCCACAGTGTCTC 630
 GlnAlaValSerAlaHisArgHisArgAlaArgValLeuLeuIleSerLysLeuSerCysValGlySerAlaIleLeuAlaThrValLeu 189
 TCCATGCCAGAGCTCTGTACAGTACCTCCAGAGGAGCAGCAGTACAGCAAGCGATGCGATGCTCTCTCATCAGAGCATGTGGAGGCC 720
 SerIleProGluLeuLeuTyrSerAspLeuGlnArgSerSerGluGlnAlaMetArgCysSerLeuIleThrGluHisValGluAla 219
 TTTATCACCATCCAGGTGGGCCAGATGGTGATCGGCTTCTGGTCCCGCTGCTGGCCATGAGCTTCTGTACCTTGTCTATCCGCACC 810
 PheIleThrIleGlnValAlaGlnMetValIleGlyPheLeuValProLeuLeuAlaMetSerPheCysTyrLeuValIleIleArgThr 249
 CTGCTCCAGGCACGCAACTTTGAGCGCAACAAGGCCATCAAGGTATCATCGCTGTGGTGGTCTTCATAGTCTTCCAGCTGCCCTAC 900
 LeuLeuGlnAlaArgAsnPheGluArgAsnLysAlaIleLysValIleIleAlaValValValPheIleValPheGlnLeuProTyr 279
 AATGGGGTGGTCTGGGCCAGCGCTGCCAAGCTTCAACATCACCAGTACGCTGTGAGCTCAGTAAGCAACTCAACATCGCCTACGAC 990
 AsnGlyValValLeuAlaGlnThrValAlaAsnPheAsnIleThrSerSerThrCysGluLeuSerLysGlnLeuAsnIleAlaTyrAsp 309
 CHO#####
 GTCACCTACAGCCTGGCCTGGCTCCGCTGCTGCGTCAACCTTCTTGTACCGCTTTCATCGGCGTCAAGTTCGGCAACGATATCTTCAAG 1080
 ValThrTyrSerLeuAlaCysValArgCysCysValAsnProPheLeuTyrAlaPheIleGlyValLysPheArgAsnAspIlePheLys 339
 CTCTCAAGGACCTGGGCTGGCTCAGCCAGGAGCAGCTCCGGCAGTGGTCTTCTGTCGGGCACATCCGGCGCTCCTCCATGAGTGTGGAG 1170
 LeuPheLysAspLeuGlyCysLeuSerGlnGluGlnLeuArgGlnTrpSerSerCysArgHisIleArgArgSerSerMetSerValGlu 369
 GCGGAGACCACCACTTCTGCCCCATAGCGACTCTTCTGCTGCACTAGAGGACCTCTCCAGGCTCCCTGGGCTGGGATAGGGA 1260
 AlaGluThrThrThrThrPheSerPro*** 375
 GCAGATGCAATGACTCAGGACATCCCCCGCCAAAAGCTGCTCAGGGGAAAAAGCAGCTCTCCCTCAGAGTGCAAGCCCTGCTCCAGA 1350
 AGATAGCTTACCCCAATCCAGCTACCTCAACCAATGCCAAAGACAGGGGTGATAAGCTAACACCCAGACACAACTGGGAAA 1440
 CAGAGCGTATTGTCCTTAAACCAAACTGAACTGAACTCCAGAACTCTTCCACCTCTCCAGCTCAACCGCCCAACGCGCTCAC 1530
 TGAAGGGCGTGGGAGTGGCTGAAGAGTCTCTGAATGAACCTTCTGGCCTCCACAGACTCAAAATGCTCAGACCACTCTTCCGAAA 1620
 ACCAGGCTTATCTCAAGACAGAGATAGTGGGGAGACTTCTGGCTTGGTAGGAAAAGCGGACATCAGTGTGTCAAACAAACTCTCT 1710
 GAACCCCTCCCTCCATCTTTTCTTCACTGTCTTCAAGCCAGCGGGAATGGCAGCTGCCACGCGCCCTAAAGCAGCACTCATCCCTC 1800
 ACTTGGCGGCTCCGCTCCAGGCTCTCAACAGGGGAGAGTGTGGTCTTCTGTCAGGCGCAGGCGAGCTGCTCCGCTGATCAAAGCCA 1890
 CACTCTGGGCTCCAGAGTGGGGATGACATGCACTCAGCTCTTGGCTCCACTGGGATGGGAGGAGGACAAAGGAAATGTCAGGGCGGG 1980
 GAGGCTGACAGTGGCGGCCAAGGCCACGAGCTTCTTCTTGTCTTGTACAGGAGTCAAAACCTCTCTCATGTTCTGCTTTCCAT 2070
 TGGTAAAGAGGCAACATTTTACCCACACAGATAAAGTTTCCCTTGAGGAAACAGCTTTAAAAAAGGAATTC 2154

FIG. 1. Nucleotide and deduced amino acid sequences of EBI 1 and EBI 2 RNAs. (A) EBI 1 has two potential translational initiation codons. In-frame stop codons are indicated by asterisks. A hydrophobic amino-terminal segment (single underline) is predicted to be a signal peptide for membrane translocation. Seven other highly hydrophobic segments are predicted to form membrane-spanning domains and are delineated by double underlines. Potential asparagine-linked glycosylation sites (CHO#####) are present in the extracellular amino-terminal segment and third extracellular loop. The sequence motif S-(I/V)-D-R-(Y/F)-X-X-X-X (where X represents consecutive hydrophobic residues) is highly conserved among a large number of G protein-coupled receptors and is indicated at the end of the third transmembrane domain (::::). (B) EBI 2 has two possible initiator methionine codons. Predicted transmembrane domains are indicated (double underlines). No signal sequence was identified.

codons at nt 34 to 36 and 46 to 48 (Fig. 1B). Although neither methionine codon is in a favored initiation context (49), the presence of an upstream in-frame termination codon and the absence of other potential open reading frames are consistent with translation initiation at the first or second methionine codon. Initiation at the first would result in a 41.2-kDa

protein. The deduced amino acid sequence predicts seven hydrophobic transmembrane segments in the characteristic configuration of a G protein-coupled receptor. In contrast to the EBI 1 protein, EBI 2 lacks a signal peptide. The amino-terminal putative extracellular domain has a potential N-linked glycosylation site. Although the EBI 2 cDNA lacks

B

GGAATTCCTGATATACACCTGGACCACCACCAATGGATATACAAATGGCAACAATTTTACTCGGCCCTCTGCAACTCCTCAGGGAAAT	90
*** METAspIleGlnMETAlaAsnAsnPheThrProProSerAlaThrProGlnGlyAsn	19
CHO#####	
GACTGTGACCTCTATGCACATCAGCAGCGCCAGGATAGTAATGCCTCTGCATTACAGCCTGGTCTTCATCATTGGGCTCCTGGGAAAC	180
AspCysAspLeuTyrAlaHisHisSerThrAlaArgIleValMetProLeuHisTyrSerLeuValPheIleIleGlyLeuValGlyAsn	49
TTACTAGCCTTGGTCTGCTATTGTTCAAAACAGGAAAAAATCAACTCTACCACCTCTATTCAACAAATTTGGTGATTCTTCATATACTT	270
LeuLeuAlaLeuValValIleValGlnAsnArgLysLysIleAsnSerThrThrLeuTyrSerThrAsnLeuValIleSerAspIleLeu	79
TTTACCACGGCTTTGGCTACACGAATAGCCTACTATGCAATGGGCTTTGACTGGAGAATCGGAGATGCCCTTGTGTAGCATAACTGGGCTA	360
PheThrThrAlaLeuProThrArgIleAlaTyrTyrAlaMetGlyPheAspTrpArgIleGlyAspAlaLeuCysArgIleThrAlaLeu	109
GTGTTTACATGAACACATATGCAGGTGTGAACCTTATGACCTGCCTGAGTATTGACCGCTTCATTGCTGTGGTGCACCTCTACGGCTAC	450
ValPheTyrIleAsnThrTyrAlaGlyValAsnPheMetThrCysLeuSerIleAspArgPheIleAlaValValHisProLeuArgTyr	139
.....	
AACAAGATAAAAGGATTGAACATGCAAAAGGGCTGTGCATATTCTCTGGATTCTAGTATTGCTCAGACACTCCCACTCCTCATCAAC	540
AsnLysIleLysArgIleGluHisAlaLysGlyValCysIlePheValTrpIleLeuValPheAlaGlnThrLeuProLeuLeuIleAsn	169
CCTATGTCAAAGCAGGAGGCTGAAAGGATTACATGCATGGAGTATCCAAACTTTGAAGAAACTAAATCTCTCCCTGGATTCTGCTTGGG	630
ProMetSerLysGlnGluAlaGluArgIleThrCysMetGluTyrProAsnPheGluGluThrLysSerLeuProTrpIleLeuLeuGly	199
GCATGTTTCATAGGATGTACTTCCACTTATAATCATTCTCATCTGCTATTCTCAGATCTGCTGCAAACTCTTCAGAACTGGCAACAA	720
AlaCysPheIleGlyTyrValLeuProLeuIleIleIleLeuIleCysTyrSerGlnIleCysCysLysLeuPheArgThrAlaLysGln	229
AACCGACTCACTGAGAAATCTGGTGTAAACAAAAAGGCTCTCAACACAATTATCTTATTATTGTTGTGTTTCTCTGTTTCACACCT	810
AsnProLeuThrGluLysSerGlyValAsnLysLysAlaLeuAsnThrIleIleLeuIleIleValValPheValLeuCysPheThrPro	259
TACCATGTTGCAATTATTCACATATGATTAAGAAGCTTCGTTTCTCTAATTTCTGGAATGTAGCCAAAGACATTGTTCCAGATTCT	900
TyrHisValAlaIleIleGlnHisMetIleLysLysLeuArgPheSerAsnPheLeuGluCysSerGlnArgHisSerPheGlnIleSer	289
CTGCACCTTACAGTATGCCTGATGAACCTCAATTGCTGCATGGACCTTTTATCTACTTCTTTGCATGTAAAGGGTATAAGAGAAAGGTT	990
LeuHisPheThrValCysLeuMetAsnPheAsnCysCysMetAspProPheIleTyrPhePheAlaCysLysGlyTyrLysArgLysVal	319
ATGAGGATGCTGAAACGGCAAGTCACTGTATCGAATTTCTAGTCTGTGAAGTCAAGCCCTGAAGAAATTCACGTGAAATGACAGAAACG	1080
MetArgMetLeuLysArgGlnValSerValSerIleSerSerAlaValLysSerAlaProGluGluAsnSerArgGluMetThrGluThr	349
GAGATGATGATACATTCCAAGTCTTCAAAATGCAAAAGTGAATGATTGTAATTTGCTTTATAGTGACGTAAACTGTATGACAAACTTTGC	1170
GlnMetMetIleHisSerLysSerSerAsnGlyLys***	361
AGGACTTCCCTTATAAAGCAAAATAATGTTTCAGCTTCCAATTAGTATTTCTTTTATTTCTTTTCATTGGGCGCTTTCCCATCTCCAAC	1260
CGGAAGTAAGCCCAAGAGAACACATAAAGCAAAACATAAAGCACAATAAAAAATGCAAAATAAATATTTCATTTTATTGTAAACGA	1350
ATACACCAAAAGGAGCGCTCTTAATAACTCCCAATGTAAAAAGTTTGTGTTTAAATAAAAAATTAATTATTCTTCCCAACAAATGGC	1440
TAGAAAGCACTGAATAGATTATATATTCGAGATGTTAATCTGTAACATCTTTTAAATAACATATTCTTAAATCCAAATTTCTCTC	1530
AATCTTAGATTAAATTCCTCAATAACACCAATGTTTGTGTTGTTCTGCTGCATAAACTTTGTTAAGCAACTCTTTTGAATA	1620
AAGAGCAGGATGCTGCGGAATTC	1644

FIG. 1—Continued.

a polyadenylate tail, a canonical polyadenylation signal (AATAAA) near the 3' end is consistent with the conclusion that the cDNA is essentially complete.

Comparison of EBI 1 and 2 with other G protein-coupled receptors. The EBI 1 and EBI 2 nucleotide and predicted amino acid sequences were compared with the GenBank (release 72 and updates), EMBL (release 31), GenBank translation, Swiss protein (release 22) and Protein Identification Resource (PIR; release 33) data bases by using the BLAST algorithm (3). EBI 1 and EBI 2 are homologous to G protein-associated receptors. EBI 1 is highly homologous to the human high- or low-affinity interleukin 8 (IL-8) receptors at both the nucleotide (data not shown) and amino acid (Fig. 2A) sequence levels. These IL-8 receptors are not expressed on lymphocytes (38, 59). Excluding the putative EBI 1 signal peptide, the overall amino acid identity among the three proteins exceeds 30%, with conservative changes observed at many of the nonidentical residues. The identity increases

to 40% when EBI 1 is compared with either IL-8 receptor individually. Additional similarities with the IL-8 receptors include a high proportion of serine and threonine near the carboxy terminus and a highly acidic amino-terminal extracellular domain. The IL-8 receptor acidic residues are implicated in binding IL-8 basic amino acids (38, 59).

The EBI 2 gene does not have such a close homolog. EBI 2 has 24% amino acid identity with the thrombin receptor (Fig. 2B; data not shown) (81). Less extensive homologies are observed with a number of other G protein-coupled receptors, including the receptors for vasoactive intestinal polypeptide, somatostatin (type 1), and angiotensin II, as well as with the low-affinity IL-8 receptor (Fig. 2B). EBI 2 also exhibits more distant homologies with EBI 1 and the high-affinity IL-8 receptor. Significantly, these are the same proteins which, in a different order, exhibit the closest homologies with the EBI 1 protein. Together they constitute a subfamily of G protein-coupled peptide receptors. The

A		
EBI 1	MDLGKPMKSVLVVALLVIFQVCLCQDEVTDDYIGDNTTVDYTLFESLCSKDKVRN	55
IL-8 RL	ME-----SDSPEDFW-----KGEDLSNYSYSTLPPLLDAAAPC-EPESE	40
IL-8 RH	MS-----NITDPQMW-----DFDDLN---FTG-MPPADEDYSPC-MLETET	36
	
TM I		
EBI 1	PKAWFLPIMYSIICFVGLLGNGLVLTYYFKRLKTMDDTYLLNLAVADILFLIT	110
IL-8 RL	INKYFVVIYALVFLLSLLGNSLVMLVILYSRVGRSVTDVYLLNLALADLLFALT	95
IL-8 RH	LNKYVVIYALVFLLSLLGNSLVMLVILYSRVGRSVTDVYLLNLALADLLFALT	91
	
TM II		
EBI 1	LPIWASAKSVVFGVHFKLIFAIYKMSFFSGMLLLLCISIDRYVAIVQAVSAH	165
IL-8 RL	LPIWASAKSVNGWIFGTFLCKVVSLLKEVNFYSGILLACISVDRYLAIVHATRTL	150
IL-8 RH	LPIWASAKSVNGWIFGTFLCKVVSLLKEVNFYSGILLACISVDRYLAIVHATRTL	146
	
TM III		
EBI 1	RHRARVLLISKLSCVGSAIATVLSIPPELLYSDDLQSSSEQAMRCSLITEHVEAF	220
IL-8 RL	TQK-RYLW--KFICLSINGLSLLALPVLFRRTVYSSNVSPACYEDMGNNTANW	202
IL-8 RH	TQK-RHLV--KFVCLGCGWGLSMNLSLPPFLPQAYHPNNSPVCYEVLGNOTAKW	198
	
TM IV		
EBI 1	-ITIQVQAMVIGFLVPLAMSFCYLVIIIRTLQARNFERNKAIVIAVVFVIV	274
IL-8 RL	RMLLRILPQSGFIVPVLIMLFCYGTTLRTLFKAHMGQKHAMRVIPAVVLIPLL	257
IL-8 RH	RMVLRILPHTFGPIVPLVFLFCYGTTLRTLFKAHMGQKHAMRVIPAVVLIPLL	253
	
TM V		
EBI 1	FQLPYNGVVLAQTVANFNITSSTCELSKQLNIAVDVYSIACVRCVNPFLYAFI	329
IL-8 RL	CWLPYNLVLLADTLMRTQVIQETCERRNHIDRALDATEILGILHSCINPLIYAFI	312
IL-8 RH	CWLPYNLVLLADTLMRTQVIQETCERRNHIDRALDATEILGILHSCINPLIYAFI	308
	
TM VI		
EBI 1	GKFRNDIFKPKDGLGCLSQEQLRWSSCRHRRSSMSVEATTITTFSP	379
IL-8 RL	GQKFRHGLLKILAIHGLISKDSLK-----DSRPSFVGSSSGHTSTTL	355
IL-8 RH	GQNFRRHGLKILAMHGLVSKFLAR-----HRVTSYT-SSSVNVSSNL	350
	
TM VII		
EBI 1	LMFLCNAIGNSLVRLTFLKYR AQAGSFDYIMMGFCNLSPLAGYLLMRLLRM	106
IL-8 RL	LMCIQGTFFENVLVITTLIYRRKKSPTSITCNLAVALDLIVVGLPFFLEYAKH	112
IL-8 RH	VVFLFGSIGNFLVIFTITWRRRIQCSGDVYFINLAAADLLFVCTLPPLMQYLL	111
	
TM VIII		
EBI 1	FEIFMNTLCKLEAFFNLISYWSPPILVFIISVLRCLLIPCATR LNVKKTLLIGQ	170
IL-8 RL	HPKLSREVVCGLNACFYICLFGVCFILNLSMDRYCVIVGVELNVRVNRKRAT	169
IL-8 RH	DHNSLASVPCTLTACFYVAMFASLCFITEIALDRYIAIV MRYRPVQKAC	169
	
TM IX		
EBI 1	WLEPAGVASCPLSVIYSSCTVGFATVALIAADRYRVLHK RTYARQSYRSTY	169

FIG. 2. Protein sequence homologies between EBI 1 and EBI 2 and previously identified G protein-coupled receptors (74). Positions of predicted transmembrane domains I through VII (TM I through TM VII) are indicated by horizontal lines above the corresponding sequences. Amino acids identical in all aligned sequences are indicated by an asterisk. Conservative changes are indicated by a dot. (A) Alignment of EBI 1 with high-affinity (IL-8 RH) and low-affinity (IL-8 RL) IL-8 receptors. (B) Alignment of EBI 2 with EBI 1 and receptors for thrombin (81), IL-8 (IL-8 RL, IL-8 HL), vasoactive intestinal polypeptide (VIP R) (71), angiotensin II (ANGT II R) (19), bovine neuropeptide Y [NY2 R (Bov)] (64), and somatostatin receptor type 1 (SOM R 1) (89). A proposed alignment with the G protein-coupled receptor homologies of herpesvirus saimiri (ECRF3) (60) and of human cytomegalovirus (HCMVUS27, HCMVUS28, and HCMVUS33) (14) is shown below.

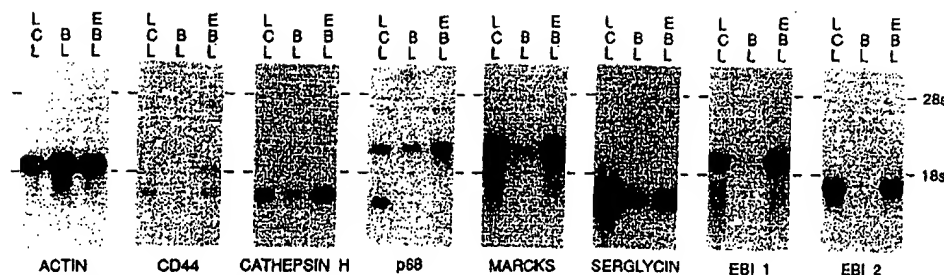


FIG. 3. RNA blot hybridization analysis of EBV-induced cellular gene expression. Polyadenylated RNA (4 to 12 μ g per lane) was size fractionated on formaldehyde-agarose gels, transferred to charged nylon membranes, and hybridized with the probes indicated at the bottom of each autoradiograph panel. RNA samples used are indicated at the top of each lane (lane LCL, EBV-immortalized primary B lymphoblastoid cell line, [IB4]; lane BL, EBV-negative BL cell line [BL41]; EB, EBV-infected BL cell line [BL41/B95-8] derived by *in vitro* infection of BL41). Dashes indicate positions of rRNA bands (18S, 28S). The band detected at 1.5 kb in lane LCL by the P68 probe is due to residual signal from a prior hybridization.

greatest conservation of residues among these proteins extends from the first transmembrane domain (Fig. 2B; TM I) to the second intracellular loop. Because of the particular conservation of an amino acid sequence among these G protein-coupled receptors, we were able to identify a new highly conserved sequence motif at the carboxy end of TM III and the adjacent second intracellular loop. This motif, S-(I/L)-D-R-(Y/F)-X-X-X-X, with X being a hydrophobic amino acid, is present in a wide variety of G protein-coupled receptors but is not present in other proteins in the data bases surveyed. Other highly conserved features of G protein-coupled receptors in EBI 1 and EBI 2 include the asparagine in TM I, the proline in TM II, the aspartate in the first intracellular loop, and the tryptophan and cysteine in the first extracellular loop. This cysteine has been postulated to be involved in disulfide linkage to a conserved cysteine present in the second extracellular loop in several other receptors, including the β -adrenergic and thrombin receptors.

Analysis of induced gene expression by RNA blot hybridization. Probes from seven of the nine EBV-induced cDNAs (vimentin and CD21 were previously shown to be EBV induced and were not further evaluated) were hybridized to identical blots of polyadenylated RNA from the EBV(+) or EBV(-) BL41 cell lines or from the EBV-transformed lymphoblastoid cell line IB4 (Fig. 3). The RNAs loaded in the EBV(+) BL41 and EBV(-) BL41 lanes were standardized with respect to β -actin reactivity. Significantly less IB4 cell RNA was used because of the high abundance of the putative induced-gene RNAs in these cells (Fig. 3, actin probe). Probes from each of the cDNA clones detected RNAs which are significantly more abundant in both IB4 and EBV(+) BL41 cells than in EBV(-) BL41 cells. Induction factors indicated in Table 1 were determined by quantitative densitometric scanning of autoradiographs and reflect the fold enhancement of signal intensities in EBV(+) BL41 cells over those in EBV(-) BL41 cells, corrected for the ratio of actin reactivities. Standardization by actin reactivity, however, significantly underestimates the absolute induction levels since actin is induced threefold by EBV infection of BL41 cells relative to glyceraldehyde phosphate dehydrogenase (data not shown) or total RNA amounts quantitated spectrophotometrically. So that the actin signal intensities would be equal, threefold more EBV(-) BL41 than EBV(+) BL41 RNA was loaded per lane. Importantly, each of the RNAs was at least as abundant relative to glyceraldehyde phosphate dehydrogenase in IB4 cells as in EBV(+) BL41 cells (Fig. 3; data not shown).

The EBI 1, EBI 2, CD44, and MARCKS genes are the most highly induced of the seven genes, being induced 21, >200, >100, and 30 times background, respectively, relative to actin (Table 1). The CD44 gene encodes three distinct RNAs of 1.6, 2.2, and 4.8 kb in both IB4 and EBV(+) BL41 cells. No CD44 RNA was detected in EBV(-) BL41 cells even after prolonged autoradiographic exposures. EBI 2 RNA was also undetectable in EBV(-) BL41 cells.

Expression of EBI 1 and EBI 2 in human cell lines and tissues. The expression of EBI 1 and EBI 2 in human cell lines and tissues was evaluated by hybridizing actin, EBI 1, or EBI 2 probes to blots of cell line or tissue RNAs. Although EBI 1 is weakly expressed in BL41, EBI 2 is not; also, neither EBI 1 nor EBI 2 is expressed in another EBV(-) BL cell line, BL30 (Fig. 4). EBI 1 and EBI 2 RNAs are abundant in primary human lymphocytes transformed by EBV *in vitro* and propagated as continuous lymphoblastoid cell lines for several years (IB4) or for less than 1 year (LCL-W91) (Fig. 4). EBI 1 RNA is faintly detectable in the human T-cell line Jurkat and is abundantly expressed in a second T-cell line, HSB-2 (Fig. 4). EBI 2 RNA is not detected in either of these T-cell lines (Fig. 4) or in a third T-cell line, Molt-4 (data not shown). EBI 1 is not expressed in the human promyelocytic line HL60, the chronic myelogenous leukemia cell line K562, the epithelial cell line RHEK-1, the fibroblast-like osteosarcoma cell line TK143, or the monocytic cell line U937 (Fig. 4). EBI 2, however, is expressed weakly, relative to actin, in HL60, U937 (U937 RNA is partially degraded), and HeLa (Fig. 4; data not shown) cells.

EBI 1 and EBI 2 RNAs are abundant in human spleen tissue, somewhat less abundant relative to actin in tonsil tissue, and undetectable in bone marrow (Fig. 4). Both genes were expressed in resting PBMCs at levels comparable to those in IB4 or LCL-W91 B lymphoblastoid cells (Fig. 4). Expression increased in parallel cultures stimulated for 72 h with pokeweed mitogen, although actin expression also increased after pokeweed mitogen treatment (Fig. 4). The EBI 1 and EBI 2 RNA in stimulated and nonstimulated PBMC cultures is likely to be mostly in B lymphocytes since EBI 1 RNA is present at low levels and EBI 2 RNA is absent in phytohemagglutinin-stimulated, PBMC-derived T lymphocytes (Fig. 4). These findings are consistent with the expression patterns observed in T-cell lines.

EBI 1 and EBI 2 RNA levels in a variety of nonhematopoietic human tissues were also evaluated. The EBI 1 probe detects small amounts of RNA in both lung and pancreas tissues (Fig. 5). Rehybridization of this blot with an Ig mu

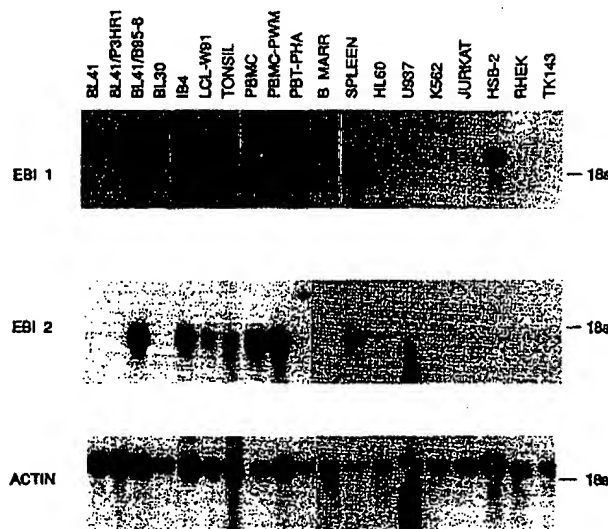


FIG. 4. Expression of EBI 1 and EBI 2 receptor genes in human lymphoid tissues and cell lines. 32 P-labeled probes indicated at the left of each panel were hybridized to blots containing RNA from the cell lines indicated at the top of each lane. BL41 and BL30 are EBV-negative BL cell lines; BL41/P3HR1 is infected with a non-transforming EBV strain, P3HR1; BL41/B95-8 is infected with a transforming EBV strain; IB4 is a cell line derived by infecting primary B lymphocytes with EBV of the B95-8 strain; LCL-W91 is a recently established cell line transformed with EBV W91; TONSIL is unfractionated cells from surgically excised human tonsils; PBMC is unfractionated peripheral blood mononuclear cells; PBMC PWM is PBMC stimulated for 72 h with pokeweed mitogen (2.5 μ g/ml); PBT PHA is T cells purified from PBMC by sheep erythrocyte rosetting, stimulated for 72 h with phytohemagglutinin (1 μ g/ml); B MARR is postmortem bone marrow; SPLEEN is unfractionated cells from surgically excised spleen; HL60 is a promyelocytic leukemia cell line; U937 is a monocytic leukemia cell line; K562 is a chronic myelogenous leukemia cell line; JURKAT is a T-cell leukemia cell line; HSB-2 is a T-cell acute lymphoblastic leukemia cell line; RHEK-1 is an adenovirus-simian virus 40-transformed human keratinocyte; TK143 is an osteosarcoma cell line. Each panel is a composite prepared from autoradiographs of two separate blots for each probe.

chain probe (Fig. 5, Ig μ probe) indicated that these tissue preparations contained significant amounts of Ig RNA, probably because of the presence of B lymphocytes in the tissues. Since EBI 1 RNA is abundant in peripheral blood lymphocytes, the EBI 1 RNA in the lung and pancreas tissues is likely to be due to B lymphocytes. Similarly, the low level of EBI 2 RNA detected in pancreas tissue is probably due to infiltrating B lymphocytes (Fig. 5). However, the abundance of EBI 2 RNA in lung tissue is too great to attribute to lymphocyte contamination and is more likely to be due to specific expression in pulmonary epithelial cells or macrophages (Fig. 5).

DISCUSSION

The present studies were undertaken to elucidate the mechanisms of EBV transformation of B lymphocytes by identifying cell genes whose expression is increased by EBV infection. By identifying genes induced in EBV(+) versus EBV(-) BL cells, the effects of EBV infection were observed in human B lymphocytes, the natural target of EBV infection and growth transformation. Differences in gene expression that were not due to EBV infection were mini-



FIG. 5. EBI 1 and EBI 2 gene expression in human tissues. EBI 1, EBI 2, and Ig μ chain (Ig μ) probes were hybridized to RNA samples from the following human tissues: heart (HE), brain (BR), placenta (PL), lung (LU), liver (LI), skeletal muscle (SM), kidney (KI), and pancreas (PA). Numbers at the left indicate positions and sizes (in kilobases) of RNA markers. Specific RNA bands are indicated by arrows to the right of each panel. The EBI 1 probe detects faint 2.4-kb bands in lung and pancreas RNA. The EBI 2 probe detects an abundant 1.9-kb band in lung RNA and a faint 1.9-kb band in pancreas RNA. The 2.7-kb Ig μ RNA is detected in lung, liver, and pancreas preparations. The 1.5-kb band in placental RNA hybridized with Ig μ probe is residual signal from a previous hybridization.

mized as a result of the isogenic background of these cells. Further, since the full repertoire of EBV genes expressed in growth-transformed B lymphocytes is expressed in the infected BL41 cells, many effects of these genes on cell gene expression are evident. Consequently, a large number of EBV-induced genes are being identified for the first time, including some novel genes likely to be mediators of B-lymphocyte growth or differentiation.

This report describes the identification of nine EBV-induced genes, CD21 and vimentin genes being the only two which were previously known to be EBV induced (8, 12). CD21 is the EBV receptor, and CD21 mRNA is induced within 36 h following in vitro infection or antigen stimulation of normal peripheral blood B cells (2, 72). EBV induction of CD21 may be a consequence of activating and differentiating effects. Gene transfer experiments in EBV(-) BL cells indicate that CD21 expression is increased by EBNA 2 or EBNA 3C expression (17, 85, 86). CD21 is also the receptor (CR2) for the C3d fragment of complement (88), associates with Ig in the B lymphocyte plasma membrane (77), and may mediate the stimulating effects of antigen-antibody and complement complexes on uninfected B lymphocytes (53).

Vimentin expression is likely to be a consequence of EBV activating effects. Vimentin is the predominant intermediate filament protein in lymphocytes and is expressed at high levels in EBV-transformed B lymphocytes (8). It associates with LMP 1 at the cell periphery (50). Infection of BL41 or BL30 cells in vitro increases vimentin mRNA and protein production (8). In gene transfer studies, LMP 1 alone can induce vimentin expression in EBV(-) Louckes or BL41 BL cells (8).

Similarly, induction of MARCKS, serglycin, cathepsin H, annexin VI, and CD44 is likely to be a consequence of EBV-activating or -differentiating effects on B-lymphocyte structures. Ig cross-linking in normal murine peripheral blood B cells results in specific phosphorylation and dramatically increases the synthesis of MARCKS protein (39, 40). Steady-state MARCKS RNA levels may be regulated in part by mRNA stability (11). The unusually high degree of homology among three prime untranslated regions of the human and bovine MARCKS RNAs (75) suggests that these sequences may play a regulatory role, possibly in mRNA stability. Although its function is unknown, MARCKS pro-

tein localizes to substratum contact points in macrophages in physical association with vinculin, talin, and cytoskeletal actin (65, 78). MARCKS protein also binds to calmodulin with high affinity and inhibits calmodulin-mediated activation of phosphodiesterase (27, 55). This interaction is disrupted by protein kinase C-mediated MARCKS phosphorylation. MARCKS-regulated interaction with calmodulin is of particular interest in light of the role of calmodulin in lymphocyte apoptosis (54) and the ability of EBV or LMP 1 to protect cells from apoptosis (35). MARCKS may be a mediator of the anti-apoptotic effects of EBV infection.

Annexin VI is a Ca^{2+} binding protein of the lipocortin family (18) and is phosphorylated in response to growth factor stimulation (43). It may associate with CD21 (6). In vitro membrane reconstitution studies indicate that annexin VI may regulate the release of Ca^{2+} from intracellular stores (34). Annexin VI is expressed only in mantle zone B cells and is not detectable in GC cells (15).

Recent experiments indicate that annexin VI plays a critical role in the formation and budding of clathrin-coated pits by a process which may be triggered by specific phosphorylation (51). These findings are of particular significance since cathepsin H and serglycin may also be intracellular vesicle constituents. Cathepsin H is a lysosomal cysteine protease (9), whereas serglycin is a core protein of proteoglycans. Although not previously known to be expressed in lymphocytes, serglycin is stored in granulocyte secretory granules, where it may neutralize hydrolytic enzymes (45, 47).

CD44 binds hyaluronic acid (4) and is the lymphocyte homing receptor for high endothelial venules. Its expression increases following anti-Ig stimulation of murine B cells (13). In contrast with other EBV-induced genes, high-level CD44 expression in vivo is observed primarily in germinal-center B lymphocytes (24). CD44 may associate with the lymphocyte cytoskeleton via an ankyrinlike molecule (42). Transfection studies with Daudi and BL41 cell lines indicate that LMP 1 is responsible for CD44 induction (85).

The most significant outcome of this study is the discovery of the first G protein-coupled peptide receptors expressed in lymphocytes exclusively (as with EBI 1) or predominantly (as with EBI 2). Both genes are expressed at high levels in EBV(+) BL cells and EBV-transformed lymphoblastoid cell lines but are expressed at low or undetectable levels in two different EBV(-) BL lines. Expression of neither gene is entirely EBV specific, and EBI 1 and EBI 2 are likely to function normally as tissue-specific mediators of polypeptide cytokine effects. EBI 1 and EBI 2 RNAs are present in PBMCs at levels comparable to their levels in lymphoblastoid cell lines; they are also detected in RNA from unfractionated tonsil tissue, which consists mostly of B lymphocytes. The much higher EBI 2 RNA level in B-lymphocyte tissues and cell lines than in phytohemagglutinin-stimulated T cells and the T-cell lines Jurkat, HSB-2, and Molt-4 is evidence that EBI 2 expression in vivo is predominantly B- rather than T-lymphocyte restricted. An intermediate level of EBI 2 RNA was observed in HL60 and U937 cell lines and in pulmonary tissue, suggesting that EBI 2 may also be expressed in monocytes, granulocytes, and pulmonary tissue in vivo. In contrast, EBI 1 is expressed in B- and T-lymphocyte lines but not in nonlymphoid tissues or cell lines; it thus appears to be entirely lymphocyte restricted. The level of EBI 1 RNA in phytohemagglutinin-stimulated peripheral blood T cells and in T-cell lines indicates that both B and T lymphocytes may contribute to the overall expression observed in unfractionated PBMC, tonsil, and spleen

cell RNA preparations. Experiments are now in progress to delineate the roles of EBI 1 and EBI 2 in normal B- and T-lymphocyte development and immune responses.

The relatively high EBI 1 and EBI 2 mRNA levels in PBMCs, spleen tissue, and, to a lesser extent, tonsil tissue is surprising in light of the low levels in BL cells. EBI 1 and EBI 2 expression may be restricted to particular stages of lymphocyte differentiation or activation. EBV(-) BL cells may correspond to a differentiation state in which expression of both genes is characteristically low. In latent EBV infection, EBV gene products may act individually or in concert to activate expression or to maintain high levels of expression in host cells which already transcribe EBI 1 and EBI 2. Preliminary data indicate that EBI 1 is induced in EBV(-) BL cells converted to LMP 1 or EBNA 2 expression by single-gene transfer. EBI 2 expression may be specifically induced by EBNA 2 or EBNA LP since expression is up-regulated in BL41 cells transfected with an EBNA LP and EBNA 2 expression vector.

The extent of EBI 1 and EBI 2 homology to G protein-coupled peptide receptors makes it probable that EBI 1 and EBI 2 are receptors which transduce ligand-binding signals through heterotrimeric GTP-binding proteins (G proteins). Homologies between EBI 1 and the high- and low-affinity IL-8 receptors are particularly striking and are apparent at both the nucleotide and amino acid levels. In fact, EBI 1 is the closest known homolog of the IL-8 receptors. The neuropeptide Y receptor type 2, for which only the bovine form has thus far been cloned, also exhibits a high degree of homology to these proteins (64). These four proteins appear to constitute a closely related subfamily within the superfamily of G protein-coupled receptors. EBI 2 exhibits more distant homology to these proteins and appears to be most closely related to the thrombin receptor.

We have identified a previously unrecognized, highly conserved G protein-coupled receptor motif at the junction of the third transmembrane domain and second intracellular loop. This motif, (A/S)-(I/V)-D-R-(Y/F)-X-X-X-X, where X represents hydrophobic residues, is characteristic of even distantly related G protein-coupled receptors, including photoreceptor opsins and receptors from phylogenetically divergent species such as *Drosophila* species. The motif is not present in other proteins. The restricted presence of this sequence in G protein-coupled receptors is most consistent with an important role in G protein-coupled receptor function. This region is implicated in receptor interaction with G proteins (48). The sequence includes five continuous hydrophobic amino acids, and ligand binding may alter the disposition of the hydrophobic portion of this sequence with respect to the adjacent plasma membrane, effecting changes which modulate G protein interaction.

Ligands for EBI 1 or EBI 2 are likely to be polypeptide cytokines since EBI 1 and EBI 2 are most closely related to G protein-coupled receptors which have polypeptide ligands. Further, a third transmembrane domain aspartate residue is highly conserved among G protein-coupled biogenic amine receptors but is absent from EBI 1 and EBI 2, excluding membership in that family (73). The close similarity between EBI 1 and the IL-8 receptors is evidence that the EBI 1 ligand is a polypeptide proinflammatory "intercrine" factor similar to ACT-2, GRO/MGSA or RANTES, or IL-8 (63).

The expected action of EBI 1 and EBI 2 is through G proteins and secondary messenger pathways by the activation of effector molecules such as adenyl cyclase, cyclic AMP phosphodiesterase, phospholipase C, or various ion

channels (20). In general, G protein-coupled peptide receptors regulate specialized functions of target cells, such as secretion of hormones in endocrine cells, membrane depolarization in neural cells, or chemotactic migration and activation of phagocytic cells. Ligand binding may also alter growth properties. Expression of the serotonin 1c receptor in immortalized rodent fibroblasts results in ligand-dependent oncogenic transformation (41). The melanoma growth-stimulatory activity protein (MGSA) binds to a G protein-coupled receptor and supports melanoma cell growth. MGSA can compete with IL-8 for binding to the IL-8 receptor, suggesting that the MGSA receptor may be closely related to the IL-8 receptors (58).

Indirect evidence indicates that a lymphocyte G protein-coupled receptor may affect phospholipase C activity and EBI 1 or EBI 2 may be the receptor that interacts in this pathway. Nonhydrolyzable GTP analogs constitutively activate both G proteins and lymphocyte polyphosphoinositide breakdown by phospholipase C (25, 33). Further, some experiments have demonstrated constitutive inhibition of phospholipase C activation by pertussis toxin, which blocks G proteins by ADP-ribosylation of their alpha subunits (25, 33, 57). Moreover, a G protein isoform regulates phospholipase C activity in brain tissue (69).

The finding that EBV induces these two putative G protein-coupled peptide receptors is also of interest in light of the recent findings that human cytomegalovirus and herpesvirus saimiri have incorporated homologs to G protein-coupled receptors into their genomes (60). Cytomegalovirus has three such genes (14). This virus is unusual among herpesviruses in its transient stimulation of cell DNA synthesis early in lytic replication and in establishing infection in precursor cells in bone marrow (52). Herpesvirus saimiri is a New World primate herpesvirus and is the herpesvirus most closely related to the EBV group of Old World primate lymphotropic herpesviruses (1). Herpesvirus saimiri can transform human T lymphocytes in vitro and can produce tumors in New World primates (7). The role of the G protein-coupled receptor homolog in herpesvirus saimiri infection has not been investigated. The finding that EBV induces G protein-coupled receptors and that cytomegalovirus and herpesvirus saimiri have incorporated G protein-coupled receptors into their genomes is compatible with the hypothesis that these G protein-coupled receptors mediate similar functions for these herpesviruses. However, comparison of the cytomegalovirus and herpesvirus saimiri homologs with EBI 1 and EBI 2 and the G protein-coupled peptide receptors reveals significant differences between the cytomegalovirus or herpesvirus saimiri G protein-coupled receptor homologs and the G protein-coupled peptide receptor group (Fig. 2B).

The identification of 9 different EBV-induced mRNAs among the first 12 clones and of 5 new and different mRNAs among 6 additional clones in various stages of sequence determination is evidence indicating that a larger group of EBV-induced genes can be identified by this approach. Other novel B-lymphocyte genes which may encode mediators of B-lymphocyte growth, differentiation, or activation or of EBV-induced growth transformation are likely to be identified.

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REFERENCES

- Albrecht, J.-C., J. Nicholas, D. Biller, K. R. Cameron, B. Biesinger, C. Newman, S. Wittmann, M. A. Craxton, H. Coleman, B. Fleckenstein, and R. W. Honess. 1992. Primary structure of the herpesvirus saimiri genome. *J. Virol.* 66:5047-5058.
- Alfieri, C., M. Birkenbach, and E. Kieff. 1991. Early events in Epstein-Barr virus infection of human B lymphocytes. *Virology*, 181:595-608. (Erratum, 185:946).
- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Aruffo, A., I. Stamenkovic, M. Melnick, C. B. Underhill, and B. Seed. 1990. CD44 is the principal cell surface receptor for hyaluronate. *Cell* 61:1303-1313.
- Baichwal, V. R., and B. Sugden. 1988. Transformation of Balb 3T3 cells by the BNLF-1 gene of Epstein-Barr virus. *Oncogene* 2:461-467.
- Barel, M., A. Gauffre, F. Lyamani, A. Fiandino, J. Hermann, and R. Frade. 1991. Intracellular interaction of EBV/C3d receptor (CR2) with p68, a calcium-binding protein present in normal but not in transformed B lymphocytes. *J. Immunol.* 147:1286-1291.
- Biesinger, B., I. Muller-Fleckenstein, B. Simmer, G. Lang, S. Wittmann, E. Platzter, R. C. Desrosiers, and B. Fleckenstein. 1992. Stable growth transformation of human T lymphocytes by herpesvirus saimiri. *Proc. Natl. Acad. Sci. USA* 89:3116-3119.
- Birkenbach, M., and A. Aderem. Unpublished observations.
- Birkenbach, M., D. Liebowitz, F. Wang, J. Sample, and E. Kieff. 1989. Epstein-Barr virus latent infection membrane protein increases vimentin expression in human B-cell lines. *J. Virol.* 63:4079-4084.
- Bohley, P., and P. O. Seglen. 1992. Proteases and proteolysis in the lysosome. *Experientia* 48:151-157.
- Boyd, A. W., K. C. Anderson, A. S. Freedman, D. C. Fisher, B. Slaughenhaupt, S. F. Schlossman, and L. M. Nadler. 1985. Studies of in vitro activation and differentiation of human B lymphocytes. I. Phenotypic and functional characterization of the B cell population responding to anti-Ig antibody. *J. Immunol.* 134:1516-1523.
- Brooks, S. F., T. Herget, J. D. Erusalimsky, and E. Rozengurt. 1991. Protein kinase C activation potentially down-regulates the expression of its major substrate, 80K, in Swiss 3T3 cells. *EMBO J.* 10:2497-2505.
- Calender, A., M. Billaud, J. P. Aubry, J. Banchereau, M. Vaillaume, and G. M. Lenoir. 1987. Epstein-Barr virus (EBV) induces expression of B-cell activation markers on in vitro infection of EBV-negative B-lymphoma cells. *Proc. Natl. Acad. Sci. USA* 84:8060-8064.
- Camp, R. L., T. A. Kraus, M. L. Birkeland, and E. Pure. 1991. High levels of CD44 expression distinguish virgin from antigen-primed B cells. *J. Exp. Med.* 173:763-766.
- Chee, M. S., S. C. Satchwell, E. Preddie, K. M. Weston, and B. G. Barrell. 1990. Human cytomegalovirus encodes three G protein-coupled receptor homologues. *Nature (London)* 344:774-777.
- Clark, D. M., S. E. Moss, N. A. Wright, and M. J. Crumpton. 1991. Expression of annexin VI (p68, 67 kDa-callectrin) in normal human tissues: evidence for developmental regulation in B- and T-lymphocytes. *Histochemistry* 96:405-412.
- Cohen, J. I., F. Wang, and E. Kieff. 1991. Epstein-Barr virus nuclear protein 2 mutations define essential domains for transformation and transactivation. *J. Virol.* 65:2545-2554.
- Cordier, M., A. Calender, M. Billaud, U. Zimmer, G. Rousset, O. Pavlish, J. Banchereau, T. Tursz, G. Bornkamm, and G. M. Lenoir. 1990. Stable transfection of Epstein-Barr virus (EBV) nuclear antigen 2 in lymphoma cells containing the EBV P3HR1 genome induces expression of B-cell activation molecules CD21 and CD23. *J. Virol.* 64:1002-1013.
- Crompton, M. R., R. J. Owens, N. F. Totty, S. E. Moss, M. D. Waterfield, and M. J. Crumpton. 1988. Primary structure of the

- human, membrane-associated Ca^{2+} -binding protein p68 a novel member of a protein family. *EMBO J.* 7:21-27. (Erratum, 7:1914.)
19. Curnow, K., I. Pascoe, and P. White. 1992. Genetic analysis of the human type 1 angiotensin II receptor. *Mol. Endocrinol.* 6:1113-1118.
 20. Dohlman, H. G., J. Thorner, M. G. Caron, and R. J. Lefkowitz. 1991. Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.* 60:653-688.
 21. Ehlin-Henriksson, B., A. Manneborg-Sandlund, and G. Klein. 1987. Expression of B-cell-specific markers in different Burkitt lymphoma subgroups. *Int. J. Cancer* 39:211-218.
 22. Fahraeus, R., A. Jansson, A. Ricksten, A. Sjoblom, and L. Rymo. 1990. Epstein-Barr virus-encoded nuclear antigen 2 activates the viral latent membrane protein promoter by modulating the activity of a negative regulatory element. *Proc. Natl. Acad. Sci. USA* 87:7390-7394.
 23. Favrot, M. C., O. Maritz, T. Suzuki, M. Cooper, I. Philip, T. Philip, and G. Lenoir. 1986. EBV-negative and -positive Burkitt cell lines variably express receptors for B-cell activation and differentiation. *Int. J. Cancer* 38:901-906.
 24. Fyfe, G., T. J. Cebra, E. Mustain, J. M. Davis, C. D. Alley, and M. H. Nahm. 1987. Subpopulations of B lymphocytes in germinal centers. *J. Immunol.* 139:2187-2194.
 25. Gold, M. R., J. P. Jakway, and A. L. DeFranco. 1987. Involvement of a guanine-nucleotide-binding component in membrane IgM-stimulated phosphoinositide breakdown. *J. Immunol.* 139:3604-3613.
 26. Gordon, J., L. Walker, G. Guy, G. Brown, M. Rowe, and A. Rickinson. 1986. Control of human B-lymphocyte replication. II. Transforming Epstein-Barr virus exploits three distinct viral signals to undermine three separate control points in B-cell growth. *Immunology* 58:591-595.
 27. Graff, J. M., T. N. Young, J. D. Johnson, and P. J. Blackshear. 1989. Phosphorylation-regulated calmodulin binding to a prominent cellular substrate for protein kinase C. *J. Biol. Chem.* 264:21818-21823.
 28. Gregory, C. D., C. F. Edwards, A. Milner, J. Wiels, M. Lipinski, M. Rowe, T. Tursz, and A. B. Rickinson. 1988. Isolation of a normal B cell subset with a Burkitt-like phenotype and transformation in vitro with Epstein-Barr virus. *Int. J. Cancer* 42:213-220.
 29. Gregory, C. D., M. Rowe, and A. B. Rickinson. 1990. Different Epstein-Barr virus-B cell interactions in phenotypically distinct clones of a Burkitt's lymphoma cell line. *J. Gen. Virol.* 71:1481-1495.
 30. Gregory, C. D., T. Tursz, C. F. Edwards, C. Tetaud, M. Talbot, B. Caillou, A. B. Rickinson, and M. Lipinski. 1987. Identification of a subset of normal B cells with a Burkitt's lymphoma (BL)-like phenotype. *J. Immunol.* 139:313-318.
 31. Guy, G. R., and J. Gordon. 1989. Epstein-Barr virus and a tumour-promoting phorbol ester use similar mechanisms in the stimulation of human B-cell proliferation. *Int. J. Cancer* 43:703-708.
 32. Harlan, D. M., J. M. Graff, D. J. Stumpo, R. L. J. Eddy, T. B. Shows, J. M. Boyle, and P. J. Blackshear. 1991. The human myristoylated alanine-rich C kinase substrate (MARCKS) gene (MACS). Analysis of its gene product, promoter, and chromosomal localization. *J. Biol. Chem.* 266:14399-14405.
 33. Harnett, M. M., and G. G. Klaus. 1988. G protein coupling of antigen receptor-stimulated polyphosphoinositide hydrolysis in B cells. *J. Immunol.* 140:3135-3139.
 34. Hazarika, P., M. A. Kaetzel, A. Sheldon, N. J. Karin, S. Fleischer, T. E. Nelson, and J. R. Dedman. 1991. Annexin VI is associated with calcium-sequestering organelles. *J. Cell. Biochem.* 46:78-85.
 35. Henderson, S., M. Rowe, C. Gregory, D. Croom-Carter, F. Wang, R. Longnecker, E. Kleff, and A. Rickinson. 1991. Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. *Cell* 65:1107-1115.
 36. Henle, G., W. Henle, and V. Diehl. 1968. Relation of Burkitt's tumor-associated herpes-type virus to infectious mononucleosis. *Proc. Natl. Acad. Sci. USA* 59:94-101.
 37. Higgins, D. G., and P. M. Sharp. 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene* 73:237-244.
 38. Holmes, W. E., J. Lee, W. J. Kuang, G. C. Rice, and W. I. Wood. 1991. Structure and functional expression of a human interleukin-8 receptor. *Science* 253:1278-1280.
 39. Hornbeck, P., H. Nakabayashi, B. J. Fowkes, W. E. Paul, and D. Kligman. 1989. A major myristylated substrate of protein kinase C and protein kinase C itself are differentially regulated during murine B- and T-lymphocyte development and activation. *Mol. Cell. Biol.* 9:3727-3735.
 40. Hornbeck, P., and W. E. Paul. 1986. Anti-immunoglobulin and phorbol ester induce phosphorylation of proteins associated with the plasma membrane and cytoskeleton in murine B lymphocytes. *J. Biol. Chem.* 261:14817-14824.
 41. Julius, D., T. J. Livelli, T. M. Jessell, and R. Axel. 1989. Ectopic expression of the serotonin 1c receptor and the triggering of malignant transformation. *Science* 244:1057-1062.
 42. Kalomiris, E. L., and L. Y. Bourguignon. 1988. Mouse T lymphoma cells contain a transmembrane glycoprotein (GP85) that binds ankyrin. *J. Cell Biol.* 106:319-327.
 43. Kenton, P., P. M. Johnson, and P. D. Webb. 1989. The phosphorylation of p68, a calcium-binding protein associated with the human syncytiotrophoblast submembranous cytoskeleton, is modulated by growth factors, activators of protein kinase C and cyclic AMP. *Biochim. Biophys. Acta* 1014:271-281.
 44. Kleff, E., and D. Liebowitz. 1990. Epstein-Barr virus and its replication, p. 1889-1920. In B. N. Fields and D. M. Knipe (ed.), its replication, Virology, 2nd ed. Raven Press, New York.
 45. Kjellen, L., and U. Lindahl. 1991. Proteoglycans: structures and interactions. *Annu. Rev. Biochem.* 60:443-475.
 46. Knutson, J. C. 1990. The level of c-fgr RNA is increased by EBNA-2, an Epstein-Barr virus gene required for B-cell immortalization. *J. Virol.* 64:2530-2536.
 47. Kolset, S. O., and J. T. Gallagher. 1990. Proteoglycans in haemopoietic cells. *Biochim. Biophys. Acta* 1032:191-211.
 48. Konig, B., A. Arendt, J. H. McDowell, M. Kahlert, P. A. Hargrave, and K. P. Hoffman. 1989. Three cytoplasmic loops of rhodopsin interact with transducin. *Proc. Natl. Acad. Sci. USA* 86:6878-6882.
 49. Kozak, M. 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* 266:19867-19870.
 50. Liebowitz, D., R. Kopan, E. Fuchs, J. Sample, and E. Kleff. 1987. An Epstein-Barr virus transforming protein associates with vimentin in lymphocytes. *Mol. Cell. Biol.* 7:2299-2308.
 51. Lin, H. S., T. C. Sudhof, and R. G. W. Anderson. 1992. Annexin VI is required for budding of clathrin-coated pits. *Cell* 70:283-291.
 52. Maciejewski, J. P., E. E. Bruening, R. E. Donahue, E. S. Mocarski, N. S. Young, and S. C. St. Jeor. 1992. Infection of hematopoietic cells by human cytomegalovirus. *Blood* 80:170-178.
 53. Matsumoto, A., J. Kopicky-Bird, R. Carter, D. Tuveson, T. Tedder, and D. Fearon. 1991. Intersection of the complement and immune systems: a signal transduction complex of the B lymphocyte containing complement receptor type 2 and CD19. *J. Exp. Med.* 173:55-64.
 54. McConkey, D. J., P. Nicotera, P. Hartzell, G. Bellomo, A. H. Wyllie, and S. Orrenius. 1989. Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic Ca^{2+} concentration. *Arch. Biochem. Biophys.* 269:365-370.
 55. McIlroy, B. K., J. D. Walters, P. J. Blackshear, and J. D. Johnson. 1991. Phosphorylation-dependent binding of a synthetic MARCKS peptide to calmodulin. *J. Biol. Chem.* 266:4959-4964.
 56. Miller, G. 1990. Epstein-Barr virus, p. 1921-1958. In B. N. Fields, and D. M. Knipe (ed.), Virology, 2nd ed. Raven Press, New York.
 57. Monroe, J. G., and S. Haldar. 1989. Involvement of a specific guanine nucleotide binding protein in receptor immunoglobulin stimulated inositol phospholipid hydrolysis. *Biochim. Biophys.*

- Acta 1013:273-278.
58. Moser, B., C. Schumacher, T. V. von Tscherner, L. I. Clark, and M. Baggiolini. 1991. Neutrophil-activating peptide 2 and gro/melanoma growth-stimulatory activity interact with neutrophil-activating peptide 1/interleukin 8 receptors on human neutrophils. *J. Biol. Chem.* 266:10666-10671.
 59. Murphy, P. M., and H. Tiffany. 1991. Cloning of complementary DNA encoding a functional human interleukin-8 receptor. *Science* 253:1280-1283.
 60. Nicholas, J., K. R. Cameron, and R. W. Honess. 1992. Herpesvirus saimiri encodes homologues of G protein-coupled receptors and cyclins. *Nature (London)* 355:362-365.
 61. Nilsson, K., and G. Klein. 1982. Phenotypic and cytogenetic characteristics of human B-lymphoid cell lines and their relevance for the etiology of Burkitt's lymphoma. *Adv. Cancer Res.* 37:319-380.
 62. Nilsson, K., G. Klein, W. Henle, and G. Henle. 1971. The establishment of lymphoblastoid lines from adult and fetal human lymphoid tissue and its dependence on EBV. *Int. J. Cancer* 8:443-450.
 63. Oppenheim, J. J., C. O. Zachariae, N. Mukaida, and K. Matsushima. 1991. Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu. Rev. Immunol.* 9:617-648.
 64. Rimland, J., W. Xin, P. Sweetnam, K. Saijoh, E. Nestler, and R. Ovman. 1991. Sequence and expression of a neuropeptide Y receptor cDNA. *Mol. Pharmacol.* 40:869-875.
 65. Rosen, A., K. F. Keenan, M. Thelen, A. C. Nairn, and A. Aderem. 1990. Activation of protein kinase C results in the displacement of its myristoylated, alanine-rich substrate from punctate structures in macrophage filopodia. *J. Exp. Med.* 172:1211-1215.
 66. Rowe, M., C. M. Rooney, C. F. Edwards, G. M. Lenoir, and A. B. Rickinson. 1986. Epstein-Barr virus status and tumour cell phenotype in sporadic Burkitt's lymphoma. *Int. J. Cancer* 37:367-373.
 67. Rowe, M., C. M. Rooney, A. B. Rickinson, G. M. Lenoir, H. Rupani, D. J. Moss, H. Stein, and M. A. Epstein. 1985. Distinctions between endemic and sporadic forms of Epstein-Barr virus-positive Burkitt's lymphoma. *Int. J. Cancer* 35:435-441.
 68. Rowe, M., D. T. Rowe, C. D. Gregory, L. S. Young, P. J. Farrell, H. Rupani, and A. B. Rickinson. 1987. Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. *EMBO J.* 6:2743-2751.
 69. Smrcka, A. V., J. R. Hepler, K. O. Brown, and P. C. Sternweis. 1991. Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. *Science* 251:804-807.
 70. Spira, G., P. Aman, N. Koide, G. Lundin, G. Klein, and K. Hall. 1981. Cell-surface immunoglobulin and insulin receptor expression in an EBV-negative lymphoma cell line and its EBV-converted sublines. *J. Immunol.* 126:122-126.
 71. Sreedhara, S., A. Robichon, and K. Peterson. 1991. Cloning and expression of the human vaso active intestinal peptide receptor. *Proc. Natl. Acad. Sci. USA* 88:4986-4990.
 72. Stashenko, P., L. M. Nadler, R. Hardy, and S. F. Schlossman. 1981. Expression of cell surface markers after human B lymphocyte activation. *Proc. Natl. Acad. Sci. USA* 78:3848-3852.
 73. Strader, C. D., I. S. Sigal, M. R. Candelore, E. Rands, W. S. Hill, and R. A. Dixon. 1988. Conserved aspartic acid residues 79 and 113 of the beta-adrenergic receptor have different roles in receptor function. *J. Biol. Chem.* 263:10267-10271.
 74. Strosberg, A. D. 1991. Structure/function relationship of proteins belonging to the family of receptors coupled to GTP-binding proteins. *Eur. J. Biochem.* 916:1-10.
 75. Stumpo, D. J., J. M. Graff, K. A. Albert, P. Greengard, and P. J. Blackshear. 1989. Molecular cloning, characterization, and expression of a cDNA encoding the "80- to 87-kDa" myristoylated alanine-rich C kinase substrate: a major cellular substrate for protein kinase C. *Proc. Natl. Acad. Sci. USA* 86:4012-4016.
 76. Suzuki, T., S. K. Sanders, J. L. Butler, G. L. Gartland, K. Komiyama, and M. D. Cooper. 1986. Identification of an early activation antigen (Bac-1) on human B cells. *J. Immunol.* 137:1208-1213.
 77. Tanner, J., J. Weis, D. Fearon, Y. Whang, and E. Kieff. 1987. Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping and endocytosis. *Cell* 50:203-213.
 78. Thelen, M., A. Rosen, A. C. Nairn, and A. Aderem. 1991. Regulation by phosphorylation of reversible association of a myristoylated protein kinase C substrate with the plasma membrane. *Nature (London)* 351:320-322.
 79. Thorley-Lawson, D. A., L. M. Nadler, A. K. Bhan, and R. T. Schooley. 1985. BLAST-2 [EBVCS], an early cell surface marker of human B cell activation, is superinduced by Epstein-Barr virus. *J. Immunol.* 134:3007-3012.
 80. Thorley-Lawson, D. A., S. L. Swendeman, C. M. Edson. 1986. Biochemical analysis suggests distinct functional roles for the BLAST-1 and BLAST-2 antigens. *J. Immunol.* 136:1745-1751.
 81. Vu, T. K., D. T. Hung, V. I. Wheaton, and S. R. Coughlin. 1991. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 64:1057-1068.
 82. Wang, D., D. Liebowitz, and E. Kieff. 1985. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell* 43:831-840.
 83. Wang, D., D. Liebowitz, and E. Kieff. 1988. The truncated form of the Epstein-Barr virus latent-infection membrane protein expressed in virus replication does not transform rodent fibroblasts. *J. Virol.* 62:2337-2346.
 84. Wang, D., D. Liebowitz, F. Wang, C. Gregory, A. Rickinson, R. Larson, T. Springer, and E. Kieff. 1988. Epstein-Barr virus latent infection membrane protein alters the human B-lymphocyte phenotype: deletion of the amino terminus abolishes activity. *J. Virol.* 62:4173-4184.
 85. Wang, F., C. Gregory, C. Sample, M. Rowe, D. Liebowitz, R. Murray, A. Rickinson, and E. Kieff. 1990. Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. *J. Virol.* 64:2309-2318.
 86. Wang, F., C. D. Gregory, M. Rowe, A. B. Rickinson, D. Wang, M. Birkenbach, H. Kikutani, T. Kishimoto, and E. Kieff. 1987. Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23. *Proc. Natl. Acad. Sci. USA* 84:3452-3456.
 87. Wang, F., S. F. Tsang, M. G. Kurilla, J. I. Cohen, and E. Kieff. 1990. Epstein-Barr virus nuclear antigen 2 transactivates latent membrane protein LMP1. *J. Virol.* 64:3407-3416.
 88. Weis, J., T. Tedder, and D. Fearon. 1984. Identification of a 145,000 M_r membrane protein as the C3d receptor (CR2) of human B lymphocytes. *Proc. Natl. Acad. Sci. USA* 81:881-885.
 89. Yamada, Y., S. Post, K. Wang, H. Tager, G. Bell, and S. Seino. 1992. Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract, and kidney. *Proc. Natl. Acad. Sci. USA* 89:251-255.

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Detection of Epstein-Barr virus DNA in cardiac and aortic tissues from chronic, active Epstein-Barr virus infection associated with Kawasaki disease-like coronary artery aneurysms

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From the Department of Pediatrics, Hokkaido University School of Medicine, Sapporo, the Pediatric Clinic, National Hakodate Hospital, Hakodate, the Department of Pediatrics, Nagoya City University Medical School, Nagoya, and the Department of Pediatrics, Gifu Prefectural Tajimi Hospital, Gifu, Japan

We describe three patients with chronic, active Epstein-Barr virus infection associated with Kawasaki disease-like coronary artery aneurysms. The Epstein-Barr virus genome was detected in three cardiac tissue samples and one aortic tissue sample examined by means of the polymerase chain reaction. These findings suggest that chronic Epstein-Barr virus infection may play a pathogenic role in the development of coronary artery aneurysms. (J PEDIATR 1993;123:90-2)

Chronic, active Epstein-Barr virus infection is characterized by fever, lymphadenopathy, splenomegaly, hepatitis, interstitial pneumonitis, and uveitis. Laboratory findings include anemia, thrombocytopenia, leukopenia, and hypergammaglobulinemia with a very high titer of EBV antibodies^{1,2}; cardiac involvement is rare. To our knowledge, our previous report of CEBV associated with coronary artery aneurysms was the first.³ We now report three patients with CEBV associated with coronary artery aneurysms, similar to the findings of Kawasaki disease.⁴

METHODS

Subjects. Three Japanese patients with CEBV and coronary artery aneurysms were studied (Table). Coronary artery aneurysms were detected by cross-section echocardiography. All patients had a chronic illness characterized by prolonged fever, coronary artery aneurysms, hepatosplenomegaly, liver dysfunction, and hypergammaglobulinemia. In addition to coronary artery aneurysms, patients 1 and 3 had dilation of the sinus of Valsalva, and patient 1 died of rupture of the sinus of Valsalva. Patients 2 and 3 also had pericarditis. These patients had several other manifestations of EBV infection: interstitial pneumonitis in patient

1, interstitial nephritis⁵ in patient 2, and rash in patient 3 (Table). None had mucous membrane involvement. The DNA was extracted from different sites in three patients with CEBV and two control cardiac tissues. Human DNA samples from Raji cells and Molt-4 cells were used as positive and negative control samples, respectively. Human embryonic fibroblast cells infected with herpes simplex virus type 1 (Seibert strain), varicella-zoster virus (wild type), and human cytomegalovirus (wild type) and human herpes

CEBV	Chronic, active Epstein-Barr virus infection
EBV	Epstein-Barr virus
KD	Kawasaki disease
PCR	Polymerase chain reaction

virus-6-infected cord blood mononuclear cells were used for these studies.

EBV serologic tests. Antibody titers to the EBV viral capsid antigen and early antigen were determined by indirect immunofluorescence. Antibody titers to EBV-determined nuclear antigen were determined by anticomplement immunofluorescence.³

Polymerase chain reaction. The PCR was performed according to a previously described method.⁶ The PCR reaction mixture consisted of 200 μ mol of each deoxyribonucleotide, 2.5 U of *Taq* DNA polymerase, 50 nmol of potassium chloride per liter, 10 mmol of Tris-HCl (pH 8.3) per liter, 1.5 mmol of magnesium chloride per liter, 0.01% (wt/vol) of gelatin, 20 pmol of each oligonucleotide primer, and 1 μ g of DNA in a volume of 100 μ l. Samples were then subjected

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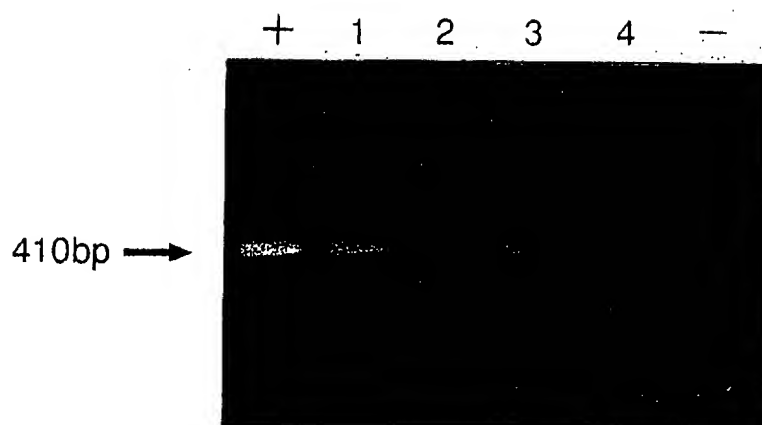


Figure. Ethidium bromide staining of PCR products. Aliquots (10 μ l) of PCR products were subjected to electrophoresis. *Lane 1*, Aortic tissue DNA from patient 2; *lane 2*, cardiac tissue DNA from patient 2; *lane 3*, cardiac tissue DNA from patient 3; *lane 4*, cardiac tissue DNA from patient 1; +, Raji cell DNA, positive control sample; -, Molt-4 cell DNA, negative control sample; bp, base pairs.

to 35 cycles of PCR, each consisting of 1 minute of denaturation at 94° C, 2 minutes of annealing at 55° C, and 3 minutes of polymerization at 72° C by a DNA thermal cycler (Perkin-Elmer Cetus, East Norwalk, Conn.). The PCR procedure was performed with primers 5' -GTGACTTCACCAAAGGTCAG-3' and 5' -TTAAAGTCCACTTACCTCTG-3'. The primers amplify and detect a 410-base-pair sequence in the region within the EBV *Bam*HI-W fragment.

Southern blot hybridization. Electrophoresis of 10 μ l of the PCR product was performed on 1.8% agarose gel. The gel was stained with 1 μ g of ethidium bromide per milliliter for 5 minutes, and the DNA bands were visualized under ultraviolet light. The PCR product was transferred onto nitrocellulose membrane filters. Each filter was hybridized with ³²P-labeled cloned fragment *Bam*HI-W of the EBV genome for 48 hours at 41° C in 1× standard saline citrate (0.15 mol sodium chloride per liter and 0.015 mol sodium citrate per liter), 50% formamide, 0.5% sodium dodecylsulfate, and heat-denatured salmon sperm DNA, 100 μ g/ml. After hybridization, the filters were washed three times at room temperature in 0.1× standard saline citrate with 0.1% sodium dodecylsulfate and then incubated three times for 1 hour at 50° C. The filters were dried and exposed to x-ray film at -80° C.⁷

RESULTS

In all these patients, EBV serologic tests revealed extremely high IgG antibody titers against early antigen and viral capsid antigen (Table). There were no significant elevations in antibody titers to other herpesviruses or to measles virus in these patients, or to adenovirus in patients 1 and 2.

Table. Clinical and laboratory findings in three patients with CEBV

	Patient No.		
	1	2	3
Age (yr)/gender	2/M	6/F	5/F
Prolonged fever	+	+	+
Coronary artery aneurysms	+	+	+
Dilation of sinus of Valsalva	+	-	+
Pericarditis	-	+	+
Rash	-	-	+
Mucous membrane involvement	-	-	-
Desquamation of fingertips	-	-	-
Lymphadenopathy	+	+	-
Hepatosplenomegaly	+	+	+
Liver dysfunction	+	+	+
Interstitial nephritis	-	+	-
Interstitial pneumonitis	+	-	-
Leukopenia	-	-	-
Anemia	+	-	+
Thrombocytopenia	-	-	-
Thrombocytosis	-	-	-
Hypergammaglobulinemia	+	+	+
EBV antibody titer			
VCA-IgG	10,240	20,480	5,120
EA-IgG	2,560	5,120	5,120
EBNA	160	40	10

VCA, Viral capsid antigen; EA, early antigen; EBNA, EBV-determined nuclear antigen.

The PCR-amplified genomic Raji cell DNA was confirmed on ethidium bromide-stained gel and by Southern blot hybridization with a radiolabeled EBV *Bam*HI-W fragment. When DNA samples from cells infected with herpes simplex virus type 1, varicella-zoster virus, human cytomegalovirus, and human herpesvirus-6 were used as

templates in the PCR reaction; no amplification was noted by direct gel analysis or Southern blot hybridization.⁶ The EBV genome, as indicated by the 410-base-pair DNA band identical to that of the positive control sample, was detected in cardiac tissue DNA from all three patients and in aortic tissue DNA from one patient (No. 2) on direct gel analysis (Figure). In two control cardiac tissue samples, the PCR procedure did not detect the EBV genome. In tissue samples from the spleen, kidney, and lung of these three patients, the PCR procedure detected the EBV genome. The EBV genome was also detected in aortic tissue of patient 2⁷ and in cardiac tissue of patient 3 by Southern blot hybridization without prior DNA amplification.

DISCUSSION

Vascular and endovascular complications are rare in EBV infection. Loeffel et al.⁸ reported an 8-year-old boy with X-linked lymphoproliferative syndrome and aneurysms of central nervous system arteries; the autopsy revealed necrotizing vasculitis in the central nervous system and microscopic changes in the coronary arteries. Ilowite et al.⁹ described a 12-year-old boy with Wiskott-Aldrich syndrome who subsequently had pulmonary vasculitis associated with EBV-induced lymphoreticular proliferation; the lung biopsy specimen showed vasculitis involving small to medium-sized arteries and veins.

We now have identified three patients with CEBV accompanied by coronary artery aneurysms. All three had high antibody titers to replicative antigens of EBV and no significant elevations in antibody titers to other herpesviruses, measles virus, or adenovirus. We were not able to test for the presence of the EBV genome in coronary arteries. However, detection of the EBV genome in the heart and aorta suggests that EBV may play a pathogenic role in the development of coronary artery aneurysms in CEBV.

Kawasaki disease is thought to be a self-limited, immunologically mediated vasculitis. We previously reported a link between KD and EBV; 49 (86%) of 57 patients with KD and 15 (68%) of 22 with recurrent KD had serologic evidence of an unusual primary EBV infection.^{10,11} The EBV genomes were identified directly by means of the PCR in peripheral blood mononuclear cell DNA samples from 21 (60%) of 35 patients within 2 weeks after the onset of KD. Furthermore, EBV genomes were also detected in all six patients who were repeatedly tested within 3 months after disease onset. In contrast, only 2 (12%) of 17 control DNA samples showed positive PCR results.⁶ Epstein-Barr virus genomes were frequently detected in peripheral blood mononuclear cell DNA from KD patients, indicating that there were much higher proportions of EBV-infected cells in their peripheral blood mononuclear cells than in those of

normal control samples. These virologic studies indicate that an unusual EBV-cell interaction may exist in KD.

A broad spectrum of associated features and complications has been reported with CEBV. Coronary artery aneurysms constitute one of the clinical features and appear not to be as rare in these patients as previously assumed. These three patients did not have the clinical hallmarks of KD but all had coronary artery aneurysms. Their recurrent episodes of fever during 6 months to several years can be distinguished from those of atypical KD.¹² All the patients with CEBV associated with coronary artery aneurysms died within 5 years of onset. We recommend periodic echocardiographic examinations for all patients with CEBV. Further studies are needed to explain the relationship between coronary artery aneurysms and EBV infection.

REFERENCES

1. Schooley RT, Carey RW, Miller G, et al. Chronic Epstein-Barr virus infection associated with fever and interstitial pneumonitis: clinical and serologic features and response to antiviral chemotherapy. *Ann Intern Med* 1986;104:636-43.
2. Straus SE. The chronic mononucleosis syndrome. *J Infect Dis* 1988;157:405-12.
3. Kikuta H, Taguchi Y, Tomizawa K, et al. Epstein-Barr virus genome-positive T lymphocytes in a boy with chronic active EBV infection associated with Kawasaki-like disease. *Nature* 1988;338:455-7.
4. Kawasaki T, Kosaki F, Okawa S, Shigematsu I, Yanagawa H. A new infantile acute mucocutaneous lymph node syndrome (MLNS) prevailing in Japan. *Pediatrics* 1974;54:271-6.
5. Koplovic J, Pinkus G, Rosen S. Interstitial nephritis in infectious mononucleosis. *Am J Kidney Dis* 1988;12:76-7.
6. Kikuta H, Nakanishi M, Ishikawa N, Konno M, Matsumoto S. Detection of Epstein-Barr virus sequences in patients with Kawasaki disease by means of the polymerase chain reaction. *Intervirology* 1992;33:1-5.
7. Kikuta H, Nakanishi M, Sakiyama Y, Matsumoto S. Chronic active Epstein-Barr virus (EBV) infection is associated with clonotypic intracellular terminal regions of the EBV. *J Infect Dis* 1989;160:546-7.
8. Loeffel S, Chang C-H, Heyn R, et al. Necrotizing lymphoid vasculitis in X-linked lymphoproliferative syndrome. *Arch Pathol Lab Med* 1985;109:546-50.
9. Ilowite NT, Fligner CL, Ochs HD, et al. Pulmonary angitis with atypical lymphoreticular infiltrates in Wiskott-Aldrich syndrome: possible relationship of lymphomatoid granulomatosis and EBV infection. *Clin Immunol Immunopathol* 1986; 41:479-84.
10. Kikuta H, Mizuno F, Osato T, et al. Kawasaki disease and an unusual primary infection with Epstein-Barr virus [Letter]. *Pediatrics* 1984;73:413-4.
11. Kikuta H, Matsumoto S, Yanase Y, Kawasaki T, Mizuno F, Osato T. Recurrence of Kawasaki disease and Epstein-Barr virus [Letter]. *J Infect Dis* 1990;162:1215.
12. Rowley AH, Gonzalez-Crussi F, Gidding SS, Duffy CE, Shulman ST. Incomplete Kawasaki disease with coronary artery involvement. *J PEDIATR* 1987;110:409-13.

Contents JULY 1993

Original articles

Development and significance of zidovudine resistance in children infected with human immunodeficiency virus 1

Mark T. Ogino, MD, Wayne M. Dankner, MD, and Stephen A. Spector, MD, San Diego, California

Resistance to zidovudine was found to be a negative prognostic factor in children infected with human immunodeficiency virus; growth and CD4⁺ cell counts were reduced, and death was more likely. Substitution or addition of a second antiretroviral drug was sometimes beneficial. *See related article on page 9.*

High-level resistance to zidovudine but not to zalcitabine or didanosine in human immunodeficiency virus from children receiving antiretroviral therapy 9

Robert N. Husson, MD, Takuma Shirasaka, MD, PhD, Karina M. Butler, MB, BCh, Philip A. Pizzo, MD, and Hiroaki Mitsuya, MD, PhD, Bethesda, Maryland

Isolates of human immunodeficiency virus type 1 were evaluated for resistance to antiviral therapy by using a selective polymerase chain reaction technique to identify mutations associated with resistance. Combination therapy did not prevent the emergence of resistance. New methods to prevent the development of resistance to nucleoside analogs are needed. *See related article on page 1.*

Prevalence of urinary tract infection in febrile infants 17

Alejandro Hoberman, MD, Han-Pu Chao, MD, David M. Keller, MD, Robert Hickey, MD, Holly W. Davis, MD, and Demetrius Ellis, MD, Pittsburgh, Pennsylvania

A study of febrile infants seen in an emergency department showed that about 5% had a urinary tract infection, regardless of whether UTI was suspected or other potential causes of fever were identified. The incidence was highest in white girls. Microscopic urinalysis was not a sensitive method of screening; urine culture was required for the diagnosis of UTI.

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Acute Epstein-Barr Virus Myocarditis Simulating Myocardial Infarction With Cardiogenic Shock

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ACUTE Epstein-Barr virus (EBV) myocarditis may yield clinical findings identical to those of acute myocardial infarction.¹⁻⁴ We describe a case of EBV myocarditis that closely resembled acute myocardial infarction complicated by cardiogenic shock.

CASE REPORT

A 38-year-old white woman went to a local hospital on Jan 7, 1986, complaining of substernal chest pain, shortness of breath, and near-syncope; an ECG was consistent with an acute anterior myocardial infarction. The patient was transferred to North Carolina Baptist Hospital for emergency cardiac catheterization.

On arrival, her systolic blood pressure was 70 mm Hg despite dopamine infusion. She appeared cyanotic, but was alert and responsive to questioning. Her oral temperature was 99.2 F (37.3 C), respiratory rate was 22/min, and pulse was thready at 150/min. Physical examination showed no jugular venous distention. Pulmonary auscultation revealed minimal bibasilar rales. Heart examination disclosed a regular tachycardia with a normal S1 and S2, and an S3 gallop. There were no murmurs. There was no hepatosplenomegaly or abdominal tenderness. The ECG showed a sinus tachycardia with 5 mm of anterior ST segment elevation and anterior Q waves (Fig 1, A). Baseline laboratory data were within normal limits except for a WBC of 16,400/cu mm with a left shift. The chest roentgenogram was normal.

On emergency cardiac catheterization, right and left coronary arteriograms were normal, but the left ventriculogram showed severe, global hypokinesia. A Swan-Ganz catheter was inserted; pulmonary artery wedge pressures averaged 24 mm Hg. The cardiac output averaged 2.5 L/min and the ejection fraction was 26%.

After catheterization, the patient was transferred to the coronary care unit for further stabilization. Additional history revealed that the patient's family had had a viral syndrome consisting of sore throat, fever, and cough during the week preceding her cardiac decompensation. Though the condition of her family members improved after several days, the patient continued to have myalgia, fever, and malaise. A severe substernal burning sensation developed, and persisted until the patient suffered the near-syncope episode that precipitated her initial emergency room visit.

The patient was managed in the coronary care unit from Jan 8 until Jan 15, 1986. She initially required hemodynamic monitoring, intravenous pressor therapy, and intra-aortic balloon counterpulsation to maintain her blood pressure, but her cardiac performance improved rapidly. On Jan 10, an endomyocardial biopsy showed widespread mononuclear inflammation, edema, focal fibrosis, and myocytic degeneration (Fig 2). The process appeared to be ten days old and was suggestive of a viral cause.

As the patient's cardiac performance improved, ECG tracings on Jan 8 and Jan 11 (Fig 1, B and C) showed resolution of the anterior ST segment elevation and the return of anterolateral R waves. An echocardiogram documented improvement in left ventricular performance. Cardiac isoenzyme determinations confirmed the presence of myocardial injury, with a peak creatine phosphokinase value of 573 U/L, with 12.3% MB fraction. The peak lactate dehydrogenase value was 679 U/L, with 26.9% LDH 1 and 26.3% LDH 2.

The patient had clinical improvement and became strong enough to walk. Serum was drawn for determination of convalescent viral titers on Jan 20, the 13th day after admission, and the patient was discharged. She returned on Feb 13 for repeat endomyocardial biopsy, which showed improvement, with few residual myocardial inflammatory cells and little residual fibrosis (Fig 3).

After the second biopsy, we reviewed the patient's microbiologic studies. Blood and urine cultures obtained during the acute phase of the illness remained negative for bacterial growth, and urine, stool, and throat cultures remained negative for viral growth. Acute viral titers were unrevealing. Convalescent titers for coxsackieviruses B1 through B6 and echoviruses 4, 9, 11, and 30 were <1:8; however, an EBV serology panel taken on Jan 20, approximately 20 days after the patient's illness began, revealed an IgG anti-EBV viral capsid antigen titer of 1:640. An anti-EBV early restricted antigen titer of 1:40 was noted, with an anti-EBV early diffuse antigen titer of <1:10; an anti-EBV nuclear antigen titer of 1:20 was also noted. These antibody titers indicated recent infection with the Epstein-Barr virus. There was no history of infectious mononucleosis or EBV infection. Follow-up EBV serologies done on April 24 showed a decrease in the IgG anti-EBV viral capsid antigen titer to 1:320. The anti-EBV early restricted antigen titer persisted at 1:40, with early diffuse titer remaining at <1:10; the anti-EBV nuclear antigen titer had increased to 1:40. Results of these studies were consistent with the expected immunologic response to a recent Epstein-Barr virus infection.

DISCUSSION

This patient had acute cardiovascular compromise after a viral prodrome consisting of sore

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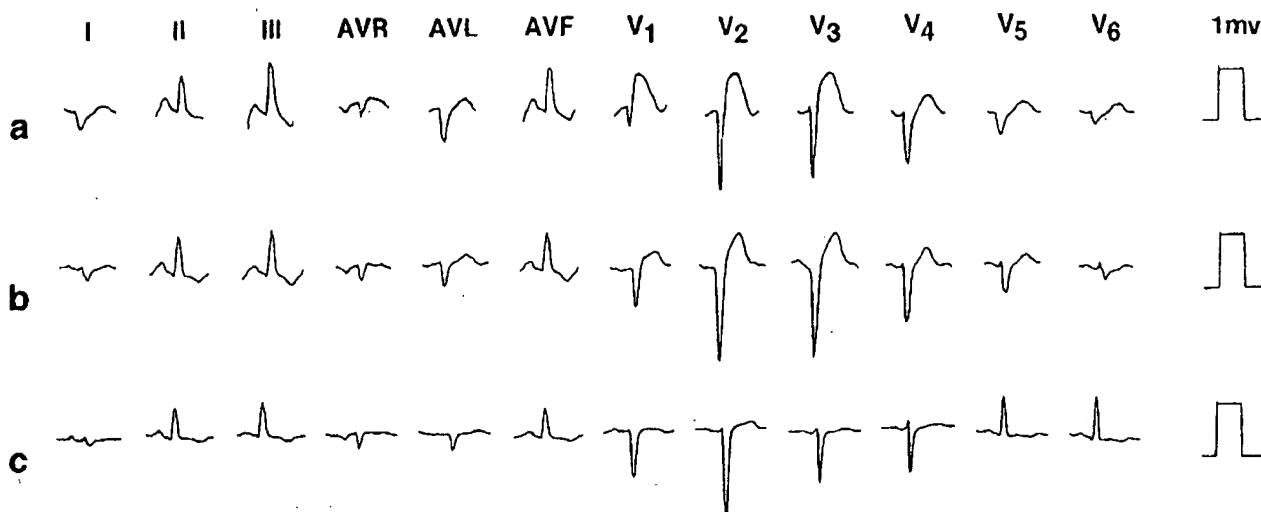


FIGURE 1. (A) Electrocardiogram at presentation on Jan 7, 1986 revealing prominent anterior ST segment elevation and pathologic Q waves. (B) Electrocardiogram Jan 11, 1986 revealing slightly lowered anterior ST segments with persistent Q waves. (C) Electrocardiogram Jan 11, 1986 revealing total resolution of anterior ST segment elevation with substantial improvement in anterolateral R wave voltage.

throat, fever, and cough. The typical findings of infectious mononucleosis, including splenomegaly, lymphadenopathy, and atypical lymphocytosis, were absent, but a specific serologic test demonstrated a recent Epstein-Barr virus infection. Most significant was the 1:40 titer of the anti-

EBV early restricted antigen. Studies have shown that "anti-early antigen responses are, as a rule, transitory," and that "the mere presence of the antibody in a serum indicates, with few exceptions, a current or recent primary Epstein-Barr virus infection."⁵ It seems likely that the Epstein-

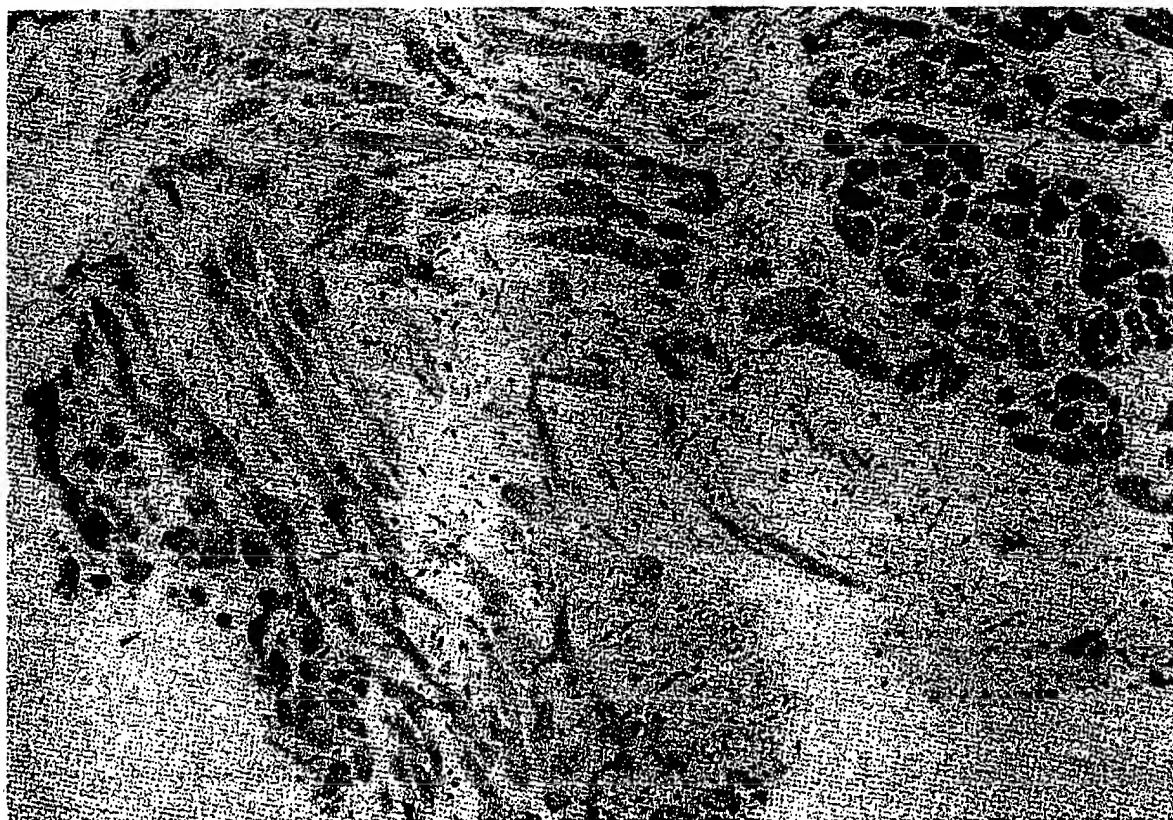


FIGURE 2. Endomyocardial biopsy on Jan 10 shows widespread mononuclear infiltration, myocytic degeneration, and focal fibrosis. (H & E, original magnification x 200)

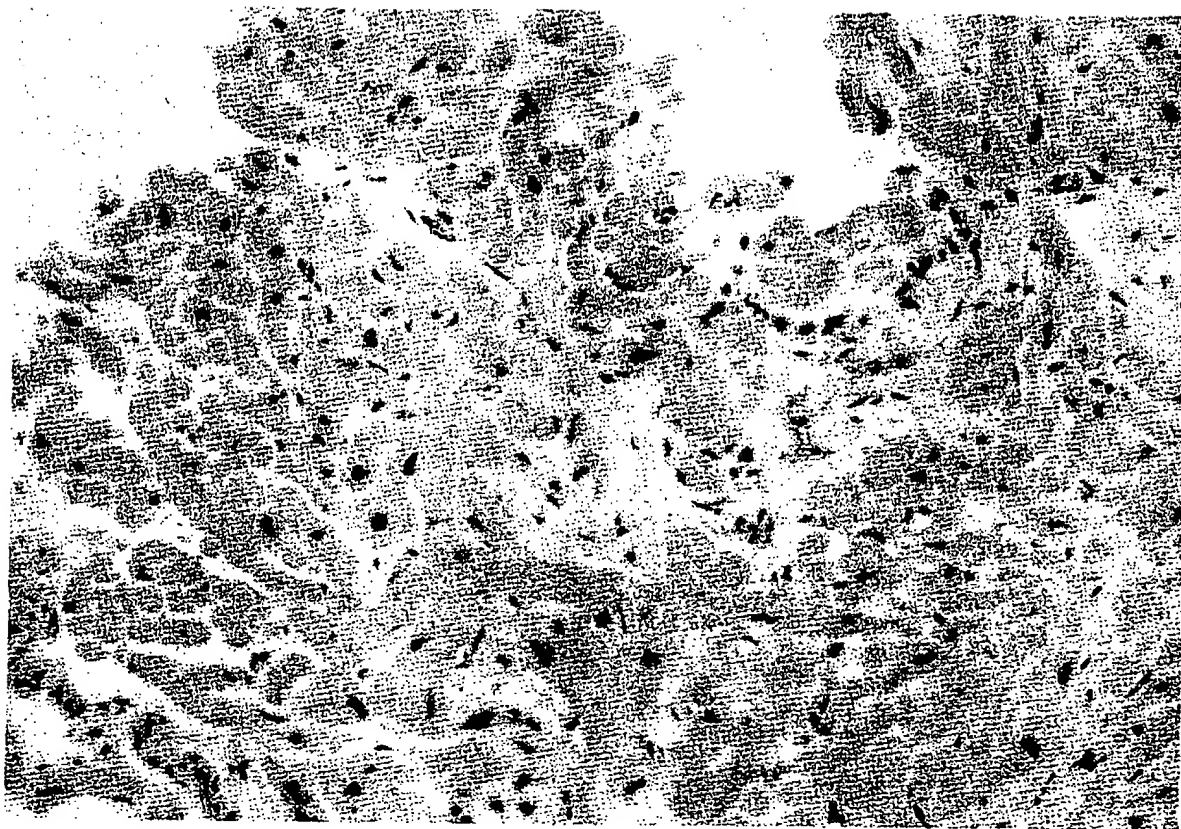


FIGURE 3. Endomyocardial biopsy on Feb 13 shows normal-appearing myocytes with little fibrosis and few residual mononuclear cells. (H & E, original magnification x 200)

Barr virus was the causative agent in this biopsy-proven case of acute myocarditis.

Initially, the clinical findings in this patient were suggestive of acute myocardial infarction. Similar cases have been reported previously. Costanzo-Nordin et al,⁶ recently reported two cases of biopsy-proven myocarditis that were initially suggestive of acute infarction. More specifically, patients diagnosed as having EBV myocarditis using clinical criteria have had initial findings suggesting acute infarction. Miller et al¹ reported the case of a 17-year-old athlete who had left-sided chest pain that radiated to the left arm. Serial ECGs were consistent with an acute inferior myocardial infarction, and the heterophil titer was positive at 1:224. When cardiac catheterization showed normal coronary arteries, his illness was attributed to EBV infection. Butler et al² described a 29-year-old man with left-sided chest pain, myalgia, and sore throat. The ECG revealed ST segment elevation in leads II, III, aV_F, V₅, and V₆. Cardiac isoenzyme levels confirmed myocardial injury, and a diagnostic rise in the anti-EBV IgG viral capsid antigen titer was documented. The patient's illness was attributed to EBV myocarditis.

Finally, EBV myocarditis documented by biopsy

has been associated with ischemic ECG changes and sudden death. Fish and Barton³ reported the case of a 20-year-old with pleuritic chest pain and cough. The heterophil titer was 1:448 and the initial ECG revealed a new right bundle branch block with inverted T waves in leads II, III, and V₁₋₄. He suffered a cardiac arrest and could not be resuscitated. Autopsy revealed inflammatory cells in the myocardium, consistent with acute myocarditis. Frishman et al⁴ described a 14-year-old girl with atypical lymphocytosis, fever, and sore throat. On the eighth day of her illness, she had cardiopulmonary arrest. Initial monitored rhythms showed ventricular fibrillation, and resuscitation was unsuccessful. Autopsy within six hours of death revealed myocardial mononuclear infiltrates, indicating recent myocarditis.

SUMMARY

We have reported the case of a 38-year-old white woman with substernal chest pain, hypotension, and ECG changes suggesting acute anterior myocardial infarction. Cardiac catheterization revealed no coronary artery pathology, but severe global hypokinesia was noted on left ventriculogram and endomyocardial biopsy revealed myocytic degeneration and mononuclear cell infiltration consistent with acute viral myocarditis.

Viral serologies confirmed a recent Epstein-Barr virus infection.

References

1. Miller R, Ward C, Amsterdam E, et al: Focal mononucleosis myocarditis simulating myocardial infarction. *Chest* 63:102-105, 1973
2. Butler T, Pastore J, Simon G, et al: Infectious mononucleosis myocarditis. *J Infection* 3:172-175, 1981
3. Fish M, Barton H: Heart involvement in infectious mononucleosis. *Arch Intern Med* 101:636-644, 1958
4. Frishman W, Kraus ME, Zabkar J, et al: Infectious mononucleosis and fatal myocarditis. *Chest* 72:535-538, 1977
5. Henle W, Henle G, Niederman JC, et al: Antibodies to early antigens induced by Epstein-Barr virus in infectious mononucleosis. *J Infect Dis* 124:58-67, 1971
6. Constanzo-Nordin MR, O'Connell JB, Subramanian R, et al: Myocarditis confirmed by biopsy presenting as acute myocardial infarction. *Br Heart J* 53:25-29, 1985

Focal Glomerulosclerosis in Hodgkin's Disease Necessitating Peritoneal Dialysis

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THE NEPHROTIC SYNDROME has been well documented as an infrequent but clinically significant complication of Hodgkin's disease.¹⁻¹³ We report a case of focal segmental glomerulosclerosis (FGS) complicated by severe nephrotic syndrome and renal failure in a young man with Hodgkin's disease.

CASE REPORT

A 16-year-old white boy saw his personal physician in May 1983 because of a two-week history of a flu-like illness and progressive generalized edema. There was no significant past medical history. Physical examination showed blood pressure of 146/100 mm Hg, bibasilar pulmonary rales, and anasarca. Serum creatinine value, BUN value, and 24-hour urine protein excretion were 2.4 mg/dl, 43 mg/dl, and 23.5 gm, respectively; one week later, they were 4.4 mg/dl, 82 mg/dl, and 30.5 gm, respectively. Creatinine clearance was 26 ml/min.

Renal biopsy revealed findings consistent with focal segmental glomerulosclerosis. There were ten glomeruli, one of which had a small segmental area with irregular thickening of the basement membrane of the peripheral capillary loops. There were focal patchy inflammatory cell infiltrates composed predominantly of lymphocytes (Fig 1). Electron microscopy showed extensive effacement of foot processes without any electron-dense deposits. Immunofluorescence study was unremarkable.

In an attempt to control proteinuria, steroids were given (three 1 gm injections of methylprednisolone sodium succinate [Solu-Medrol], followed by 60 mg of prednisone every other day for three weeks). Hemodialysis via a percutaneous

femoral catheter was required on two occasions to control uremia and intravascular volume. After one month of unsuccessful treatment, including a trial of indomethacin, a peritoneal catheter was placed and the patient was trained to perform continuous ambulatory peritoneal dialysis. The therapeutic goal was to use the dialysis to control edema and diminish proteinuria by decreasing urine volume. Figures 2 and 3 detail the daily urine volume, creatinine clearance, protein excretion, and serum albumin and globulin values. Dialysis protein excretion was 2.3 gm on one day during the seventh week of treatment. Other than occasional nausea and one episode of peritonitis due to *Staphylococcus epidermidis*, the patient tolerated dialysis without significant morbidity.

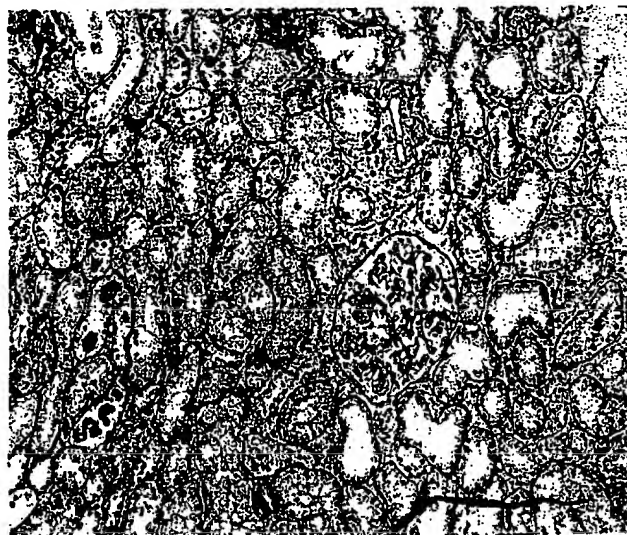


FIGURE 1. Glomerulus showing sclerotic segment. (PAM, original magnification x 100)

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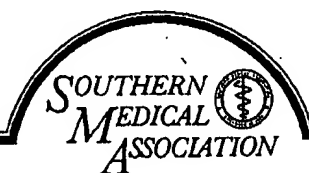
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Central role of the P2Y₁₂ receptor in platelet activation

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Platelet activation occurs in response to vessel injury and is important for the arrest of bleeding. Platelet activation during disease states leads to vascular occlusion and ischemic damage. The P2Y₁₂ receptor, activated by ADP, plays a central role in platelet activation and is the target of P2Y₁₂ receptor antagonists that have proven therapeutic value.

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The vessel wall contains a continuous lining of endothelium that serves as a barrier between the circulating platelets and the prothrombotic subendothelial matrix (1). Upon vessel injury, the endothelial layer is disrupted and the circulating platelets are exposed to subendothelial proteins such as vWF, collagen, and vitronectin, among others (1). The platelets initially interact with the subendothelium through adhesive receptors, such as GPIb-IX-V receptors, that mediate rolling and tethering of the platelets to vWF at the site of vascular injury.

Next, the platelet collagen receptors $\alpha_2\beta_1$ and GPVI mediate a more firm adhesion and cause further platelet activation. These initial interactions with the subendothelium cause the release of contents from the platelet dense granules, which contain platelet agonists such as ADP, and the α -granules, which contain fibrinogen, factor V, and P-selectin (1). The release of the granule contents causes further platelet activation, but it also fuels the coagulation response as a result of the release of factor V and fuels the inflammatory response through the exposure of P-selectin on the platelet surface. The platelet also generates lipid mediators such as thromboxane A₂. ADP elicits its effects on the platelet through the P2Y₁ and P2Y₁₂ receptors (2), whereas thromboxane A₂ activates the thromboxane-prostanoid (TP) receptor on the platelet surface (1). The released dense granule contents cause further platelet activation and recruitment of circulating platelets to the site of injury. Platelets interacting with these mediators also

undergo platelet shape change, a process of actin cytoskeletal reorganization that changes the platelets from a disc shape to a round shape with long, filopodial extensions that form a meshwork of platelets in the platelet plug (3). Also, tissue factor is exposed, which initiates the coagulation response that results in formation of thrombin. Thrombin activates platelets via interactions with the proteinase-activated receptor-1 (PAR1) and PAR4 receptors (4) and also cleaves fibrinogen to form fibrin. Fibrin further stabilizes the accumulating platelet plug at the site of injury, resulting in a stable hemostatic plug.

Interactions of the platelets with collagen, vWF, ADP, thromboxane A₂, and thrombin cause intracellular platelet signaling that leads to the activation of the heterodimeric integrin $\alpha_{IIb}\beta_3$, also known as the fibrinogen receptor (5). The intracellular platelet signaling from these agonists causes the fibrinogen receptor to change from a low-affinity state to a high-affinity state that binds fibrinogen (6). Fibrinogen binds to the platelets via the activated fibrinogen receptor, and this cross-linking of platelets to fibrinogen results in platelet aggregates that accumulate and arrest bleeding at the site of injury (Figure 1). Thus, platelet activation is the product of many signals originating from many receptors, which each contribute to the formation of a platelet plug.

Pathophysiologic conditions, such as atherosclerotic plaque rupture, can lead to aberrant platelet activation resulting in arterial thrombosis, which can cause myocardial infarction and ischemic stroke (6). The importance of ADP in this process has been demonstrated both by antiplatelet drugs that target the P2Y₁₂ receptor (2) and by patients with dysfunctional P2Y₁₂ receptors (7). Antagonism of the P2Y₁₂ receptor with either ticlopidine or clopidogrel is clinically effective in the prevention of myocardial infarction, ischemic stroke, and vascular death (8). Despite the established role of the P2Y₁₂ receptor in the hemostatic response,

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Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: proteinase-activated receptor (PAR); 2-methylthio-ADP (2-MeSADP).

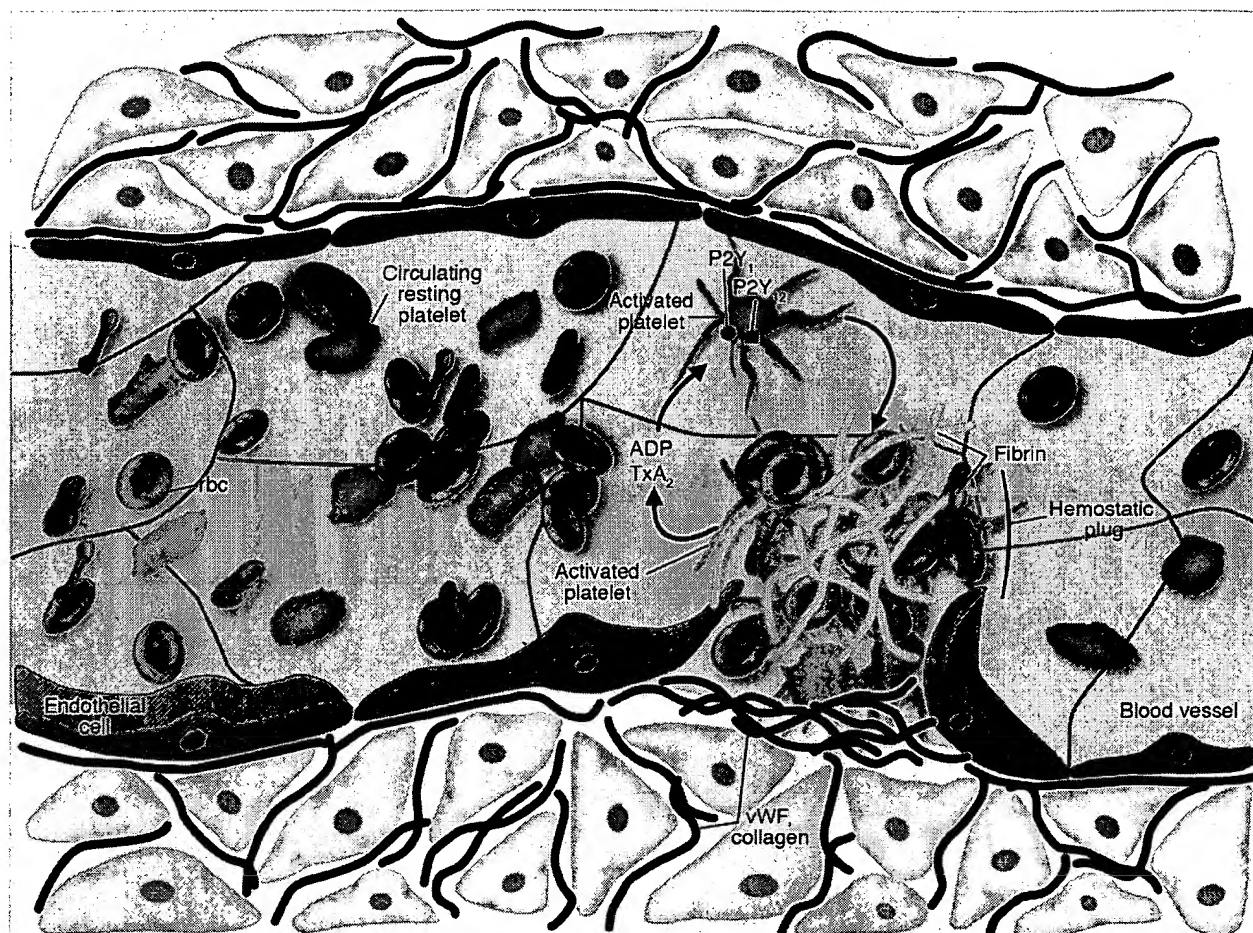


Figure 1

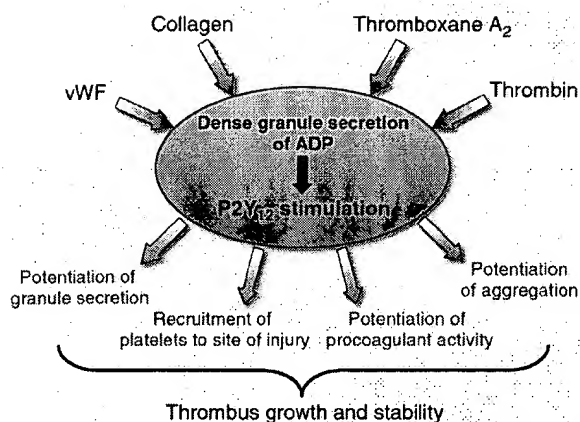
The hemostatic process. Upon vessel injury, platelets roll and become tethered to the vessel wall by interactions with vWF and collagen (noted as black strands). These interactions cause platelet shape change, and release of ADP from dense granules. The activated platelet also generates thromboxane A₂ (TxA₂). Both ADP and TxA₂ are agonists that cause further platelet activation and accumulation of platelets at the site of injury. Vessel injury also causes exposure of tissue factor, which catalyzes the coagulation response. This response results in the formation of thrombin, which further activates platelets and cleaves fibrinogen to form fibrin. The combination of activated platelets and fibrin at the site of injury forms a stable hemostatic plug that arrests bleeding.

the full implications of P2Y₁₂ receptor antagonism in the prevention of thrombosis remain incompletely understood. It is hoped that more clinically effective P2Y₁₂ antagonists will prevent the incidence of ischemic events that stem from aberrant platelet activation and therefore will be used as improved and suitable treatments for thrombosis.

The central role of the P2Y₁₂ receptor: ex vivo effects

Prior to the cloning of the P2Y₁₂ receptor, drugs that selectively target this receptor had been widely used as antiplatelet agents (2). Ex vivo studies used platelets treated with clopidogrel or reversible antagonists of the P2Y₁₂ receptor and led to the conclusion that the P2Y₁₂ receptor is crucial to several platelet functions. Thus far, studies have identified a potentiating role for the P2Y₁₂ receptor in dense granule secretion (9), fibrinogen-receptor activation (10–14), and, as reported in a recent issue of the *JCI*, thrombus formation (15, 16),

identifying it as a central mediator of the hemostatic response. This receptor is also important for the irreversible platelet aggregation induced not only by ADP, but also by thromboxane A₂ and the PAR1-selective peptide agonist SFLLRN (17, 18). The P2Y₁₂ receptor also causes inhibition of stimulated adenylyl cyclase (19, 20) but does not play any role in ADP-induced platelet shape change and intracellular calcium mobilization (17, 18). Furthermore, G_i signaling that is mediated by P2Y₁₂ receptor activation can lead to platelet aggregation when either G_q or G_{12/13} pathways are simultaneously stimulated (10, 11, 14), or by itself when exposed to high concentrations of ADP (100 μM) (21). The P2Y₁₂ receptor plays a crucial role in ADP-mediated generation of thromboxane A₂, another important platelet activator (22). Signaling events downstream of the P2Y₁₂ receptor also potentiate agonist-induced dense granule release and procoagulant activity (17). In addition, α-granule release and subsequent expression of P-selectin on activated platelets



depend on P2Y₁₂ activation (23, 24). Interestingly, all the functions of the P2Y₁₂ receptor can be mimicked by epinephrine, which stimulates members of the G_i family of G proteins by binding to the platelet α_{2A} receptor (17). Thus, the P2Y₁₂ receptor plays a central role in platelet activation, in the recruitment of other platelets to the site of injury subsequent to the adhesion of platelets to vWF and collagen, and in the enhancement of the efficiency of platelet activation by other agonists such as thrombin and thromboxane A₂, which are generated as secondary platelet agonists (Figure 2).

The P2Y₁₂ receptor couples primarily to G α_{i2} and less prominently to other members of the G_i family, resulting in the inhibition of adenylyl cyclase (25). Epinephrine, through stimulation of the α_{2A} receptor and resulting G_z signaling, also achieves the same effect (26). However, reduced levels of cAMP are not directly responsible for the downstream effects of P2Y₁₂ receptor activation (25, 26). G_i signaling leads to activation of PI3K, Akt, Rap1b, and potassium channels (17). Mice lacking PI3K- γ show aberrations in platelet function only when low doses of ADP are used, but are provided protection from thromboembolism (27). Recent studies indicate that Rap1b, Akt, and potassium channels are important functional effectors downstream of P2Y₁₂ receptor stimulation (28–31) (Figure 3).

Patients with defective P2Y₁₂ receptor function

Patients with a defect in the gene encoding the P2Y₁₂ receptor have a congenital bleeding disorder (7, 32–35). A number of patients have been identified that have

Figure 2

The central role of the P2Y₁₂ receptor in platelet activation. Exposure of platelets to vWF and collagen results in the adhesion of platelets and subsequent release of ADP from dense granules. Similarly, activation of platelets by thrombin or thromboxane A₂ also results in release of ADP. Secreted ADP activates the P2Y₁₂ receptor (as depicted) and the P2Y₁ receptor (not shown). P2Y₁₂ receptor activation likely affects thrombus growth and stability by recruiting platelets to the site of injury, and by potentiating dense granule release, procoagulant activity, and aggregation.

decreased aggregation responses to ADP and to low doses of other agonists such as collagen and thrombin (32–35). These patients generally have normal platelet shape change responses to ADP but have impaired abilities to inhibit adenylyl cyclase activity (33). While patients with defective P2Y₁₂ receptor function have dense granules that are normal in both numbers and content, platelet release of granules is generally decreased because of the potentiating effects of the P2Y₁₂ receptor on granule secretion (34). Patients and mice lacking functional P2Y₁₂ receptors have increased bleeding times (7, 32, 36). Patients who are heterozygous for the P2Y₁₂ receptor bind intermediate amounts of 2-methylthio-ADP (2-MeSADP), an agonist of the P2Y₁ and P2Y₁₂ receptors, and also have extended bleeding times (7, 34, 37). Some patients with P2Y₁₂ receptor deficiency have been shown to possess a truncated form of the receptor due to deletions in the gene, whereas other individuals have mutations that lead to impaired P2Y₁₂ receptor function (38). Analysis of the P2Y₁₂ receptor sequence from a patient with impaired ADP responses has identified amino acid residues important in P2Y₁₂ receptor function (35). A G-to-A mutation in one allele changed Arg256 into Gln, while a C-to-T alteration resulted in Arg265 changing to Trp (35). Though receptor number

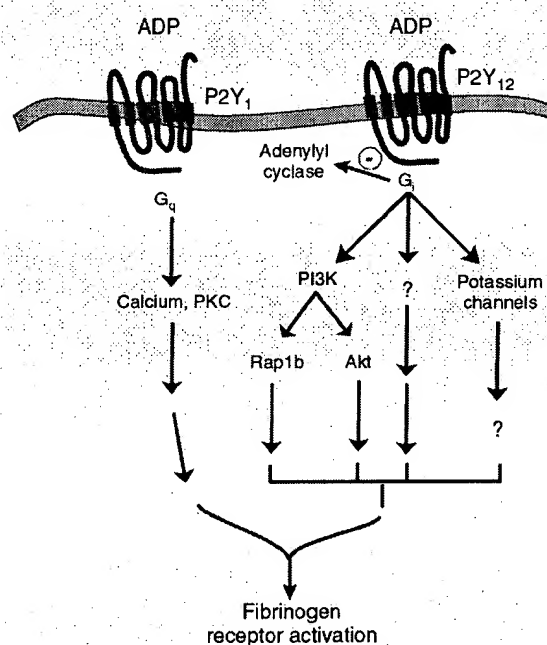


Figure 3

Intracellular signaling events downstream of the P2Y₁ and P2Y₁₂ receptors. ADP binds to the P2Y₁₂ receptor and causes a number of intracellular signaling events downstream of the G_i pathway that contribute to fibrinogen receptor activation and platelet aggregation. The P2Y₁₂ receptor-mediated inhibition of adenylyl cyclase is not directly responsible for fibrinogen receptor activation. Potassium channels and PI3K are also activated by the P2Y₁₂ receptor. Both Rap1b and Akt are signaling mediators that contribute to platelet aggregation and are activated in a PI3K-dependent manner. Other mediators of P2Y₁₂ signaling remain to be elucidated.

Table 1
In vivo effects of P2Y₁₂ receptor blockade

Species	P2Y ₁₂ blockade	Injury/stimulus	Embolization	Bleeding time	In vivo thrombus characteristics	Ref.
Rabbit (mesenteric arterioles)	AR-C69931MX	Mechanical injury	↓	Microvessel bleeding time unchanged	Size of thrombus reduced	15
Mouse (mesenteric artery)	P2Y ₁₂ ^{-/-}	Ferric chloride, tail bleeding	↑	Tail bleeding time extended, ↑ time to occlusion	Delayed, unstable, nonocclusive thrombus	16
Mouse	P2Y ₁₂ ^{+/-}	Ferric chloride, tail bleeding	Unchanged from wild type	Unchanged from wild type	ND	16
Human	Homozygous	-	ND	Extended from normal	ND	32-35
Human	Heterozygous	-	ND	Extended from normal	ND	34

The role of the P2Y₁₂ receptor in thrombus formation and its effect on bleeding times have been characterized using pharmacologic antagonists, mice deficient in the P2Y₁₂ receptor, and patients who have a mutation or truncation of the P2Y₁₂ receptor. Multiple species and models of thrombosis have been used to characterize the effect of P2Y₁₂ blockade in vivo, leading to varying conclusions and interpretations. ND, not determined.

and affinity for 2-MeSADP were unchanged, these mutations were demonstrated to lead to impaired G_i signaling in response to P2Y₁₂ stimulation (35). Expressed P2Y₁₂ receptors containing these mutations had a similar loss of function (35). Thus, these residues play an important role in the function of the P2Y₁₂ receptor but have no effect on the binding of ADP.

Bleeding times

The P2Y₁₂ receptor antagonist clopidogrel has been shown to be efficacious when occupying even 50% of the P2Y₁₂ receptor population (39); thus an antagonist of the P2Y₁₂ receptor could be an effective therapeutic even when 50% of the P2Y₁₂ receptors are functioning. Patients deficient in the P2Y₁₂ receptor, such that their platelets bind only intermediate levels of 2-MeSADP, have slightly prolonged bleeding times (7, 34, 37). The P2Y₁₂ receptor has been cloned, and mice that are deficient in the P2Y₁₂ receptor have been generated. Consistent with observations in patients deficient in the P2Y₁₂ receptor, mice lacking the P2Y₁₂ receptor have increased tail bleeding times (19, 36, 40, 41). However, heterozygous mice show little or no change in bleeding times (16). It is currently unclear why a 50% decrease in the population of functional P2Y₁₂ receptors, either following clopidogrel treatment or in humans heterozygous for the P2Y₁₂ receptor, prolongs bleeding time, while heterozygous mice show little change in bleeding time (Table 1).

Role of the P2Y₁₂ receptor in shear-induced platelet activation

Pharmacologic blockade of the P2Y₁₂ receptor in physiologic conditions of arterial flow revealed that this receptor is essential for platelet aggregation under shear conditions. Blood from a patient with defective P2Y₁₂ receptors, or normal blood treated with the reversible P2Y₁₂ receptor antagonist AR-C69931MX, exhibited small and loosely packed thrombi, whereas normal individuals formed large, densely packed thrombi in physiologic flow experiments (42). P2Y₁₂

antagonism also decreased shear-induced platelet aggregation; however, greater inhibition was achieved by antagonism of both the P2Y₁₂ and P2Y₁ receptors (43). P2Y₁₂ antagonism has also been shown to decrease P-selectin expression and microparticle formation that is initiated by platelet interactions with vWF (44). Thus, the P2Y₁₂ receptor also plays a role in the formation of platelet aggregates under shear conditions and contributes to thrombus formation on surfaces coated with either collagen or vWF.

Clinical implications of P2Y₁₂ blockade

Consistent with the central role of the P2Y₁₂ receptor in thrombosis, P2Y₁₂ receptor antagonists reduce occlusive thrombosis in animal models. Clinical studies using clopidogrel demonstrate a significantly reduced risk of peripheral artery disease, myocardial infarction, ischemic stroke, or vascular death, in comparison with aspirin therapy (8). Combination therapy with both clopidogrel and aspirin has been shown by the CURE (Clopidogrel in Unstable Angina to Prevent Recurrent Ischemic Events) study to result in enhanced beneficial effects, and this has led to FDA approval of clopidogrel for the treatment of some acute coronary syndromes (45). An ongoing trial for the Management of Atherothrombosis with Clopidogrel in High-Risk Patients with Recent Transient Ischemic Attack or Ischemic Stroke (MATCH) is expected to test the effectiveness and safety of combined clopidogrel plus aspirin therapy versus clopidogrel only in patients who have experienced a transient ischemic attack or ischemic stroke (46). The results of this trial will further clarify the efficacy of combination therapy. Despite its beneficial effects, clopidogrel has been shown to cause the development of the immune-mediated syndrome thrombotic thrombocytopenic purpura (47).

The benefits of P2Y₁₂ antagonism have been validated by multiple clinical trials, but some of the characteristics of the present P2Y₁₂ antagonists could be improved. The metabolism of clopidogrel to its active

form is required before the onset of drug action is achieved, and an interaction with the cholesterol-lowering drug atorvastatin, which also requires hepatic metabolism by cytochrome P450 3A4, has been identified (48, 49). Post hoc analysis of clinical studies where patients received both clopidogrel and atorvastatin found beneficial effects of clopidogrel in both the presence and the absence of atorvastatin, suggesting that the interaction between the two pharmacologic agents does not alter the clinical effect of clopidogrel (50). Another study also found no difference in the clinical outcome of patients taking clopidogrel with atorvastatin or with other statins (51). The clopidogrel metabolite irreversibly blocks P2Y₁₂ receptor function for the lifespan of the platelet. Hence, prior to surgery, clearance of clopidogrel-treated platelets is necessary to prevent bleeding complications. Thus, new research efforts are aimed at discovering faster-acting, reversible P2Y₁₂ receptor antagonists that would allow more control over antiplatelet treatments.

In vivo analysis of thrombus growth and stability

With the established role of the P2Y₁₂ receptor as the central point of thrombus formation, in vivo analysis will clarify the mechanism by which the P2Y₁₂ receptor contributes to thrombus growth and stability. Multiple studies have begun to characterize the effects of either P2Y₁₂ antagonism or P2Y₁₂ knockout on the formation of a thrombus using various models of vessel injury. A recent report by van Gestel et al. (15) investigated the effect of the P2Y₁₂ receptor antagonists AR-C69931MX and clopidogrel on in vivo thrombus growth and stability (Table 1). Mechanical injury of mesenteric arterioles in rabbits treated with AR-C69931MX or clopidogrel resulted in decreased thrombus height (20% reduction) within the vessel but no change in the bleeding time of the vessel (15). P2Y₁₂ receptor blockade significantly reduced the total duration of embolization with fewer and smaller emboli being produced, and it also reduced the size of the initial thrombus without affecting its stability (15). Thrombin generation was decreased in the AR-C69931MX-treated mice, suggesting that the P2Y₁₂ receptor contributes to the procoagulant response (15). In clopidogrel-treated mice, thrombosis scores were significantly reduced compared with those in controls (52). In addition, there was a delay in the time of initial thrombus formation in the clopidogrel-treated mice (52). Treatment with AR-C69931MX also decreased the reocclusion rate and improved myocardial tissue perfusion in a canine model of coronary electrolytic injury (53). Andre et al. (16) used several approaches to explore the effects of P2Y₁₂ receptor deficiency on thrombus formation. FeCl₃-induced vessel wall injury of the mouse mesenteric artery resulted in occlusion of the wild-type mouse vessel; however, the P2Y₁₂-deficient mouse vessel remained unoccluded in eight out of nine mice (16). The appearance of the first thrombus was delayed and only small "unstable" thrombi formed in P2Y₁₂^{-/-} mice (16). There was

increased embolization from the thrombus of P2Y₁₂-deficient mice (16). Contrary to the observations of van Gestel et al. (15), more embolization occurred in the P2Y₁₂^{-/-} mice compared with wild-type or heterozygous mice. The differences between these studies are likely due to differences in both species (rabbit versus mouse) and experimental parameters (mechanical injury versus FeCl₃ injury) and represent the beginning of our understanding of the effects of P2Y₁₂ antagonism on thrombus formation in vivo.

Data on the role of the P2Y₁₂ receptor in platelet activation and thrombus formation suggest that this receptor is important for the potentiation of many platelet responses and for the formation of a stable hemostatic plug. The tools available for the analysis of P2Y₁₂ receptor function have facilitated characterization of the implications of P2Y₁₂ antagonism, though the studies performed thus far also raise more questions. The differences in embolization between homozygous and heterozygous mice observed by Andre et al. (16) raise several important questions. Would complete blockade of the P2Y₁₂ receptor be a better therapeutic goal, and would it be more beneficial than clopidogrel because of abolished receptor function? Why do results differ depending on the use of a P2Y₁₂ receptor antagonist as opposed to the use of P2Y₁₂ receptor-knockout mice? Is the reason for the increased bleeding times observed in patients and mice lacking the P2Y₁₂ receptor due to unstable thrombus formation or the small size of the thrombus? What are the reasons for differences in embolization in the studies thus far? Answers to these questions are important because, ultimately, definition of the central role of the P2Y₁₂ receptor would translate into the development of a more effective antithrombotic agent, and the answers would affect treatment modalities. The models for thrombus formation have provided varying results regarding the effect of P2Y₁₂ antagonism and/or knockout on thrombus size and embolization. The method of vessel injury, vessel size, and species differences must be considered in the interpretation of the effects of P2Y₁₂ antagonism on thrombus formation. Of course, it is the clinical data that will determine the effectiveness of such therapies, but it is the models of thrombosis that will guide the efforts toward new and improved P2Y₁₂ antagonists. Thus, the large amount of data obtained using clopidogrel treatment, and the studies of van Gestel et al. (15) and Andre et al. (16), may herald the beginning of a new era in antithrombotics.

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1. Jackson, S.P., Nesbitt, W.S., and Kulkarni, S. 2003. Signaling events underlying thrombus formation. *J. Thromb. Haemost.* 1:1602-1612.
2. Kunapuli, S.P., et al. 2003. ADP receptors: targets for developing antithrombotic agents. *Curr. Pharm. Des.* 9:2303-2316.
3. Paul, B.Z., et al. 2003. Dynamic regulation of microtubule coils in ADP-induced platelet shape change by p160ROCK (Rho-kinase). *Platelets.* 14:159-169.

4. Kahn, M.L., Nakanishi-Matsui, M., Shapiro, M.J., Ishihara, H., and Coughlin, S.R. 1999. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J. Clin. Invest.* **103**:879–887.
5. Brass, L.F., Manning, D.R., Cichowski, K., and Abrams, C.S. 1997. Signaling through G proteins in platelets: to the integrins and beyond. *Thromb. Haemost.* **78**:581–589.
6. Shattil, S.J., Kashiwagi, H., and Pampori, N. 1998. Integrin signaling: the platelet paradigm. *Blood*. **91**:2645–2657.
7. Cattaneo, M., and Gachet, C. 1999. ADP receptors and clinical bleeding disorders. *Arterioscler. Thromb. Vasc. Biol.* **19**:2281–2285.
8. 1996. A randomized, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. *Lancet*. **348**:1329–1339.
9. Dangelmaier, C., Jin, J., Smith, J.B., and Kunapuli, S.P. 2001. Potentiation of thromboxane A2-induced platelet secretion by Gi signaling through the phosphoinositide-3 kinase pathway. *Thromb. Haemost.* **85**:341–348.
10. Jin, J., and Kunapuli, S.P. 1998. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc. Natl. Acad. Sci. U. S. A.* **95**:8070–8074.
11. Dorsam, R.T., Kim, S., Jin, J., and Kunapuli, S.P. 2002. Coordinated signaling through both G12/13 and G(i) pathways is sufficient to activate GPIIb/IIIa in human platelets. *J. Biol. Chem.* **277**:47588–47595.
12. Quinton, T.M., et al. 2002. Protein kinase C- and calcium-regulated pathways independently synergize with Gi pathways in agonist-induced fibrinogen receptor activation. *Biochem. J.* **368**:535–543.
13. Kauffenstein, G., et al. 2001. The P2Y₁₂ receptor induces platelet aggregation through weak activation of the α (IIb) β (3) integrin: a phosphoinositide 3-kinase-dependent mechanism. *FEBS Lett.* **505**:281–290.
14. Nieswandt, B., Schulte, V., Zywiec, A., Gratacap, M.P., and Offermanns, S. 2002. Costimulation of Gi- and G12/G13-mediated signaling pathways induces integrin α IIb β 3 activation in platelets. *J. Biol. Chem.* **277**:39493–39498.
15. van Gestel, M.A., et al. 2003. In vivo blockade of platelet ADP receptor P2Y₁₂ reduces embolus and thrombus formation but not thrombus stability. *Arterioscler. Thromb. Vasc. Biol.* **23**:518–523.
16. Andre, P., et al. 2003. P2Y₁₂ regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. *J. Clin. Invest.* **112**:398–406. doi:10.1172/JCI200317864.
17. Kunapuli, S.P., Dorsam, R.T., Kim, S., and Quinton, T.M. 2003. Platelet purinergic receptors. *Curr. Opin. Pharmacol.* **3**:175–180.
18. Gachet, C. 2001. ADP receptors of platelets and their inhibition. *Thromb. Haemost.* **86**:222–232.
19. Hollopeter, J., et al. 2001. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature*. **409**:202–207.
20. Zhang, F.L., et al. 2001. ADP is the cognate ligand for the orphan G protein-coupled receptor SP199. *J. Biol. Chem.* **276**:8608–8615.
21. Ohlmann, P., et al. 2000. ADP induces partial platelet aggregation without shape change and potentiates collagen-induced aggregation in the absence of Galphaq. *Blood*. **96**:2134–2139.
22. Jin, J., Quinton, T.M., Zhang, J., Rittenhouse, S.E., and Kunapuli, S.P. 2002. Adenosine diphosphate (ADP)-induced thromboxane A₂ generation in human platelets requires coordinated signaling through integrin α (IIb) β (3) and ADP receptors. *Blood*. **99**:193–198.
23. Zhao, L., Bath, P., and Heptinstall, S. 2001. Effects of combining three different antiplatelet agents on platelets and leukocytes in whole blood in vitro. *Br. J. Pharmacol.* **134**:353–358.
24. Storey, R.F., Judge, H.M., Wilcox, R.G., and Heptinstall, S. 2002. Inhibition of ADP-induced P-selectin expression and platelet-leukocyte conjugate formation by clopidogrel and the P2Y₁₂ receptor antagonist AR-C69931MX but not aspirin. *Thromb. Haemost.* **88**:488–494.
25. Daniel, J.L., Dangelmaier, C., Jin, J., Kim, Y.B., and Kunapuli, S.P. 1999. Role of intracellular signaling events in ADP-induced platelet aggregation. *Thromb. Haemost.* **82**:1322–1326.
26. Yang, J., et al. 2000. Loss of signaling through the G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. *Proc. Natl. Acad. Sci. U. S. A.* **97**:9984–9989.
27. Hirsch, E., et al. 2001. Resistance to thromboembolism in PI3Kgamma-deficient mice. *FASEB J.* **15**:2019–2021.
28. Chrzanowska-Wodnicka, M., Schoenwaelder, S., and White, G.C., II. 2003. Rap1b regulates integrin IIb3 activity and platelet function: lessons from a knockout. *Blood*. **102**:773a. (Abstr.)
29. Shankar, H., et al. 2003. Role of G-protein coupled inwardly rectifying potassium channels (GIRKs) in P2Y₁₂ receptor-mediated platelet functional responses. *Blood*. **102**:774a. (Abstr.)
30. Woulfe, D.S., et al. 2003. Mice lacking Akt2 have defects in platelet granule secretion and fibrinogen binding and reduced thrombus formation in vivo. *Blood*. **102**:207a. (Abstr.)
31. Kim, S., Jin, J., and Kunapuli, S.P. 2004. Akt activation in platelets depends on Gi signaling pathways. *J. Biol. Chem.* In press.
32. Nurden, P., et al. 1995. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. Its influence on glycoprotein IIb-IIIa complex function. *J. Clin. Invest.* **95**:1612–1622.
33. Cattaneo, M., Lecchi, A., Randi, A.M., McGregor, J.L., and Mannucci, P.M. 1992. Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. *Blood*. **80**:2787–2796.
34. Cattaneo, M., Lecchi, A., Lombardi, R., Gachet, C., and Zighetti, M.L. 2000. Platelets from a patient heterozygous for the defect of P2CYC receptors for ADP have a secretion defect despite normal thromboxane A₂ production and normal granule stores: further evidence that some cases of platelet 'primary secretion defect' are heterozygous for a defect of P2CYC receptors. *Arterioscler. Thromb. Vasc. Biol.* **20**:E101–E106.
35. Cattaneo, M., et al. 2003. Molecular bases of defective signal transduction in the platelet P2Y₁₂ receptor of a patient with congenital bleeding. *Proc. Natl. Acad. Sci. U. S. A.* **100**:1978–1983.
36. Foster, C.J., et al. 2001. Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *J. Clin. Invest.* **107**:1591–1598.
37. Cattaneo, M., et al. 1997. Deficiency of (P-33)2MeS-ADP binding sites on platelets with secretion defect, normal granule stores and normal thromboxane A₂ production. Evidence that Adp potentiates platelet secretion independently of the formation of large platelet aggregates and thromboxane A₂ production. *Thromb. Haemost.* **77**:986–990.
38. Conley, P.B., and Delaney, S.M. 2003. Scientific and therapeutic insights into the role of the platelet P2Y₁₂ receptor in thrombosis. *Curr. Opin. Hematol.* **10**:333–338.
39. Bennett, J.S. 2001. Novel platelet inhibitors. *Annu. Rev. Med.* **52**:161–184.
40. Savi, P., et al. 2001. P2Y₁₂, a new platelet ADP receptor, target of clopidogrel. *Biochem. Biophys. Res. Commun.* **283**:379–383.
41. Takasaki, J., et al. 2001. Molecular cloning of the platelet P2T(AC) ADP receptor: pharmacological comparison with another ADP receptor, the P2Y₁ receptor. *Mol. Pharmacol.* **60**:432–439.
42. Remijn, J.A., et al. 2002. Role of ADP receptor P2Y₁₂ in platelet adhesion and thrombus formation in flowing blood. *Arterioscler. Thromb. Vasc. Biol.* **22**:686–691.
43. Turner, N.A., Moake, J.L., and McIntire, L.V. 2001. Blockade of adenosine diphosphate receptors P2Y₁₂ and P2Y₁ is required to inhibit platelet aggregation in whole blood under flow. *Blood*. **98**:3340–3345.
44. Goto, S., Tamura, N., Eto, K., Ikeda, Y., and Handa, S. 2002. Functional significance of adenosine 5'-diphosphate receptor (P2Y₁₂) in platelet activation initiated by binding of von Willebrand factor to platelet GPIIb/IIIa induced by conditions of high shear rate. *Circulation*. **105**:2531–2536.
45. Mitrka, M. 2001. Results of CURE trial for acute coronary syndrome. *JAMA*. **285**:1828–1829.
46. Hacke, W. 2002. From CURE to MATCH: ADP receptor antagonists as the treatment of choice for high-risk atherothrombotic patients. *Cerebrovasc. Dis.* **13**:22–26.
47. Bennett, C.L., et al. 2000. Thrombotic thrombocytopenic purpura associated with clopidogrel. *N. Engl. J. Med.* **342**:1773–1777.
48. Clarke, T.A., and Waskell, L.A. 2003. The metabolism of clopidogrel is catalyzed by human cytochrome P450 3A and is inhibited by atorvastatin. *Drug Metab. Dispos.* **31**:53–59.
49. Lau, W.C., et al. 2003. Atorvastatin reduces the ability of clopidogrel to inhibit platelet aggregation: a new drug-drug interaction. *Circulation*. **107**:32–37.
50. Saw, J., et al. 2003. Lack of adverse clopidogrel-atorvastatin clinical interaction from secondary analysis of a randomized, placebo-controlled clopidogrel trial. *Circulation*. **108**:921–924.
51. Wienbergen, H., et al. 2003. Comparison of clinical benefits of clopidogrel therapy in patients with acute coronary syndromes taking atorvastatin versus other statin therapies. *Am. J. Cardiol.* **92**:285–288.
52. Lenain, N., Freund, M., Leon, C., Cazenave, J.P., and Gachet, C. 2003. Inhibition of localized thrombosis in P2Y₁-deficient mice and rodents treated with MRS2179, a P2Y₁ receptor antagonist. *J. Thromb. Haemost.* **1**:1144–1149.
53. Wang, K., et al. 2003. Blockade of the platelet P2Y₁₂ receptor by AR-C69931MX sustains coronary artery recanalization and improves the myocardial tissue perfusion in a canine thrombosis model. *Arterioscler. Thromb. Vasc. Biol.* **23**:357–362.

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